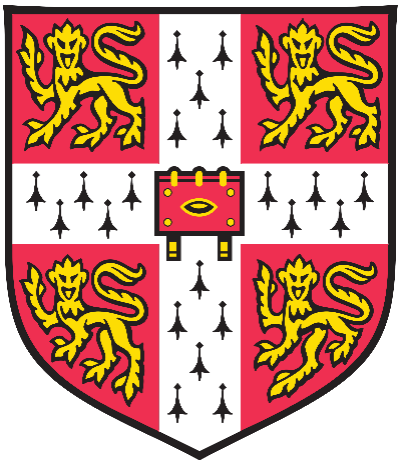

Investigating the role of IgG and Fcγ receptors in intestinal inflammation

Tomas Castro Dopico

Christ's College, University of Cambridge

Supervisor: Dr. Menna R. Clatworthy

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This dissertation is submitted for the degree of Doctor of Philosophy

Preface

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration, except as declared in the Preface or specified in the text.

This dissertation is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text.

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This dissertation does not exceed the prescribed word limit and is approximately 52,000 words in length.

Tomas Castro Dopico

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Contents

1. Introduction 1
1.1. The immune system 1
1.2. Antibodies 4
1.3. Fcγ receptors 11
1.4. Fcγ receptors and disease 22
1.5. The gastrointestinal tract and inflammatory bowel disease 28
1.6. Immunopathology in inflammatory bowel disease 35
1.7. Intestinal B cells 44
1.8. The intestinal macrophage system 51
1.9. Innate lymphoid cells 59
1.10. Hypotheses and aims of PhD 68
2. Methods 70
2.1. Mice 70
2.2. *In vivo* procedures 71
2.3. Tissue sample preparation 73
2.4. Flow cytometry 75
2.5. *In vitro* stimulation assays 78
2.6. Immunofluorescence 79
2.7. RNA analysis 80
2.8. Soluble protein assays 81
2.9. RNAseq and microarray analyses (with John Ferdinand) 82
2.10. Statistics 86
3. IgG and FcγR signalling in intestinal inflammation 87
3.1. Introduction and hypotheses 87
3.2. IgG within the gastrointestinal tract 87
3.3. Profiling intestinal B cell populations in intestinal inflammation 92
3.4. Fcγ receptor signalling in intestinal inflammation 94
3.5. Tissue-resident immune cell FcγR expression 97
3.6. FcγR-associated inflammatory networks in intestinal inflammation 101
3.7. Immune complex stimulation of intestinal leukocytes 107
3.8. Discussion 111
4. Fcγ receptor signalling drives IL-1β-dependent intestinal inflammation 119
4.1. Introduction and hypotheses 119
4.2. Transcriptomic analysis of FcγR signalling in intestinal macrophages 120
4.3. Manipulation of the A/I ratio using FcγRIIB-transgenic mouse models 126
4.4. Chronic DSS-induced colitis in *Fcgr2b*-deficient mice 127
4.5. Chronic DSS-induced colitis in macrophage transgenic mice 145

4.6. Discussion 151
5. Expression and function of Fcγ receptors on group 3 innate lymphoid cells 160
5.1. Introduction and hypotheses 160
5.2. Profiling FcγR expression on innate lymphoid cells 161
5.3. Immune complex stimulation of ILC3s *in vitro* 164
5.4. Analysis of canonical FcγR signalling in ILC3s 166
5.5. The role of FcγR signalling on ILC3 antigen presentation 167
5.6. Modulation of ILC3 FcγR expression during inflammation 169
5.7. ILC3 activation in *Fcgr2b*-deficient mice 170
5.8. Global transcriptional profiling of IgG-stimulated ILC3s 173
5.9. Discussion 180
6. ILC-macrophage crosstalk mediates intestinal immune defence 187
6.1. Introduction and hypotheses 187
6.2. ILC depletion in *Citrobacter rodentium* infection 188
6.3. ILC depletion in DSS-induced colitis 193
6.4. Potential mechanisms for macrophage regulation by ILCs 196
6.5. Chemokine expression by intestinal ILCs 197
6.6. Cytokine analysis in ILC-depleted colons 198
6.7. *In vitro* macrophage GM-CSF stimulation 200
6.8. GM-CSF neutralisation in DSS-induced colitis 202
6.9. Potential mechanisms for GM-CSF-mediated macrophage activation 204
6.10. IL-1β-mediated cross-talk between macrophages and ILCs 207
6.11. Discussion 209
7. Conclusion and future directions 216
7.1. IgG and FcγR signalling in intestinal inflammation 216
7.2. The function of FcγRs on ILC3s 218
7.3. ILC-macrophage crosstalk mediates intestinal immune defence 220
8. Acknowledgements 222
9. Appendix 223
10. References 224

Abbreviations

ABMR	Antibody-mediated rejection
AHR	Aryl hydrocarbon receptor
A/I	Activating-to-inhibitory ratio
AID	Activation-induced cytidine deaminase
AMP	Anti-microbial peptide
AOM	Azoxymethane
APC	Antigen presenting cell
APRIL	A proliferation-inducing ligand
ARNT	Aryl hydrocarbon receptor nuclear translocator
BAFF	B cell activating factor
BCR	B cell receptor
BMDC	Bone marrow-derived dendritic cell
BMDM	Bone marrow-derived macrophage
BMP2	Bone morphogenic protein 2
Breg	Regulatory B cell
BSA	Bovine serum albumin
C	Constant
CAI	Clinical activity index
CD	Crohn's disease
CDR	Complementarity determining region
cDSS	Chronic dextran sodium sulfate-induced colitis
CFU	Colony forming unit
CHILP	Common helper innate lymphoid progenitor
CIA	Collagen-induced arthritis
CLP	Common lymphoid progenitor
CNV	Copy number variation
CRP	C-reactive protein
cRMPI	Complete RPMI
CSR	Class-switch recombination
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
DSA	Donor-specific antibody
DSS	Dextran sodium sulfate
DTT	Dithiothreitol
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
EILP	Early innate lymphoid progenitor
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FcγR	Fcγ receptor
FcRn	Neonatal Fc receptor
FCS	Fetal calf serum
FDC	Follicular dendritic cell
GALT	Gut-associated lymphoid tissue
GC	Germinal centre
GF	Germ-free
GI	Gastrointestinal
GlcNAc	*N*-acetylglucosamine
GM-CSF	Granulocyte macrophage-colony stimulating factor
GSEA	Gene-set enrichment analysis
GWA	Genome wide association
H	Heavy

HC	Healthy control
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HHC	Household healthy control
HIF-1α	Hypoxia-inducible factor 1 alpha
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HSP90	90-kDa heat shock protein
IBD	Inflammatory bowel disease
IC	Immune complex
IEC	Intestinal epithelial cell
IL	Interleukin
IL-1Ra	IL-1 receptor antagonist
IL-22BP	Interleukin-22 binding protein
ILC	Innate lymphoid cell
ILC1	Group 1 innate lymphoid cell
ILC2	Group 2 innate lymphoid cell
ILC3	Group 3 innate lymphoid cell
ILCP	Innate lymphoid cell progenitor
ILF	Isolated lymphoid follicle
Ig	Immunoglobulin
ITAM	Immunoreceptor tyrosine-based activating motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
ITP	Immune-mediated thrombocytopenic purpura
IVIG	Intravenous immunoglobulin
J	Joining
KO	Knockout
L	Light
LI	Large intestine
LN	Lymph node
LP	Lamina propria
LPMC	Lamina propria mononuclear cell
Ltα1β2	Lymphotoxin-α1β2
LTi	Lymphoid tissue inducer
M-CSF	Macrophage-colony stimulating factor
MDDC	Monocyte-derived dendritic cell
MDM	Monocyte-derived macrophage
MG	Mitotracker green
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
MMP	Matrix metalloprotease
MNP	Mononuclear phagocyte
MS	Multiple sclerosis
M-TG	Macrophage-trangenic
MTOR	Mammalian target of rapamycin
MTORC1	Mammalian target of rapamycin complex 1
MZ	Marginal zone
NCR	Natural killer cell receptor
NK	Natural killer
NLRP3	Nucleotide-binding oligomerisation domain, leucine-rich repeat and pyrin domain containing 3
NMS	Normal mouse serum
NO	Nitric oxide
N-TG	Non-transgenic
O	Ovalbumin
O-IC	Ovalbumin immune complex

OR	Odds ratio
OSM	Oncostatin M
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCA	Principle component analysis
PCR	Polymerase chain reaction
PGE2	Prostaglandin E2
pIgR	Polymeric Ig receptor
PMN	Polymorphonuclear
PP	Peyer's patch
PRR	Pattern recognition receptor
qPCR	Quantitative polymerase chain reaction
RA	Rheumatoid arthritis
ROS	Reactive oxygen species
RtA	Retinoic acid
SAP	Serum amyloid protein
SC	Secretory component
SCFA	Short chain fatty acid
SFB	Segmented filamentous bacteria
SHM	Somatic hypermutation
SI	Small intestine
S-IgA	Secretory-immunoglobulin A
SLE	Systemic lupus erythematosus
SLO	Secondary lymphoid organ
SNP	Single nucleotide polymorphism
STAT	Signal transducer and activator of transcription
SYK	Spleen tyrosine kinase
TCR	T cell receptor
TD	T cell-dependent
TF	Transcription factor
Tfh	T follicular helper
Th	T helper
Th1	T helper 1
Th2	T helper 2
Th17	T helper 17
TI	T cell-independent
TL1A	Tumour necrosis factor-like ligand 1A
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNF	Tumour necrosis factor
TNFR1	Tumour necrosis factor receptor 1
TNFR2	Tumour necrosis factor receptor 2
TNFSF15	Tumour necrosis factor superfamily member 15
Treg	Regulatory T cell
TRUC	*T-bet*$^{-/-}$ x *Rag*$^{-/-}$ ulcerative colitis
UC	Ulcerative colitis
V	Variable
WT	Wild type

Abstract

IgA is the dominant antibody isotype found at mucosal surfaces during homeostasis. However, genetic variation in Fcγ receptors (FcγRs), a family of receptors that mediate immune cell activation by IgG, influences susceptibility to inflammatory bowel disease (IBD), suggesting that IgG may be important during gut inflammation. IBD is a chronic relapsing condition with two major subtypes, Crohn's disease (CD) and ulcerative colitis (UC), both driven by aberrant immune responses to commensals. In the first part of this thesis, we sought to investigate anti-commensal IgG responses in patients with UC and to determine the mechanism by which local IgG might contribute to intestinal inflammation.

We found that UC and murine dextran sodium sulfate (DSS)-induced colitis are associated with a significant increase in anti-commensal IgG and local enrichment of FcγR signalling pathway genes. The genes most robustly correlated with *FCGR2A*, an activating FcγR associated with UC susceptibility, were *IL1B* and *CXCL8*. *Ex vivo* stimulation of human and murine lamina propria mononuclear cells with IgG immune complexes (IC) resulted in an increase in these cytokines/chemokines. *In vivo* manipulation of the macrophage FcγR A/I ratio in transgenic mice determined IL-1β and Th17 cell induction. Finally, IL-1β blockade in mice with a high FcγR A/I ratio reduced IL-17 and IL-22-producing T cells and the severity of colitis. Our data reveal that commensal-specific IgG contributes to intestinal inflammation via FcγR-dependent, IL-1β-mediated Th17 activation.

In this thesis, we have also addressed the interplay between IgG and group 3 innate lymphoid cells (ILC3s). ILC3s are closely related to natural killer cells, which are known to express FcγRs, and are characterised by their production of Th17 cytokines. Here, we have shown that ILC3s express FcγRs, that ICs drive IL-22 production and MHC class II expression by ILC3s, and FcγR signalling induces a transcriptional programme that reinforces ILC3 maintenance and functionality. These results represent a new paradigm for ILC activation, with direct regulation by the adaptive immune response.

Finally, we have begun to address the role played by ILC3-derived cytokines in the regulation of local tissue-resident immune cells. We have demonstrated that ILC depletion significantly alters the activation state of intestinal macrophages, resulting in detrimental bacterial outgrowth following *C. rodentium* infection but protection from overwhelming DSS-induced inflammation. We have shown that GM-CSF promotes macrophage IL-1β and IL-23 production, which in turn act to reinforce ILC3-derived GM-CSF and IL-22 secretion *in vitro*, respectively. Therefore, ILC3s are essential coordinators of the local inflammatory response within the gut through activation and possible recruitment of immune cells, and their modulation may be beneficial in the treatment of IBD.

1. Introduction

1.1. The immune system

1.1.1. Overview

The immune system is essential for protective responses against potentially pathogenic microorganisms and for the elimination of tumours. Furthermore, a growing body of literature demonstrates fundamental roles for immune cells in the maintenance of homeostatic physiology and host-commensal mutualism at barrier sites throughout the body. However, dysregulated immunity has detrimental consequences and may give rise to autoimmune diseases, such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), as well as inflammatory disorders, including inflammatory bowel disease (IBD), amongst others [1].

The immune response can be divided into two major categories: innate and adaptive (Fig. 1.1). Innate immunity comprises rapid nonspecific defences, sufficient for initial protection against most microorganisms. These are driven by the recognition of conserved pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) expressed on innate immune cells, such as macrophages, dendritic cells (DCs), and neutrophils. Adaptive immunity is driven by two major cell populations, T cells and B cells, that express somatically-recombined antigen-specific receptors. Driven by antigen encounter and signals derived from the innate arm, the activation of adaptive immunity results in highly targeted and augmented immune responses and the generation of immunological memory. Presented here is a brief overview of the cells that make up these two arms.

1.1.2. Innate immunity

The epithelium at barrier sites, such as the gastrointestinal (GI) tract, provides the first defence against the invasion of microorganisms. As well as a physical barrier, epithelial cells can directly sense microorganisms through PRRs and instruct local immune cells through the production of inflammatory mediators, including cytokines and chemokines [2]. Following breach of the epithelium, tissue-resident innate cells are the first to sense PAMPs or danger-associated molecular patterns (DAMPs) released by damaged cells.

Haematopoietic innate immunity consists of a variety of myeloid and lymphoid cells (Fig. 1.1A). Tissue-resident macrophages act as sentinels and display voracious phagocytic capabilities for the elimination of microorganisms. These cells also orchestrate the initiation, progression, and resolution of inflammatory responses through their production of cytokines and chemokines [3]. Macrophage function is complemented by granulocytes, such as neutrophils and eosinophils. Neutrophils are myeloid cells rapidly recruited to sites of inflammation that express an array of cell surface receptors. They respond rapidly and potently to a wide range

of pathogenic stimuli and display essential microbicidal activities, such as the production of reactive oxygen species (ROS) and inflammatory mediators [4].

DCs sit at the interface of innate and adaptive immunity. Their primary function is to transport antigen from peripheral sites to draining lymph nodes in a CCR7-dependent manner for major histocompatibility complex (MHC)-dependent presentation to T cells [5], [6]. Additionally, DC-derived cytokines play key roles in shaping immune responses. This includes the differentiation of T cell subsets or the induction of B cell class-switch recombination (CSR), as shall be discussed shortly [7]–[10].

Finally, distinct lymphocyte subsets also form part of innate immunity. Natural killer (NK) cells are a class of cytotoxic lymphocyte characterised by the production of perforin, granzymes, and interferon gamma (IFNγ) in response to ligation of an array of cell surface receptors. These include cytokine receptors, as well as those for cellular antigens exposed on stressed and infected cells [11]. In addition, the recently describe helper innate lymphoid cells (ILCs) are enriched at mucosal sites, where they receive inflammatory stimulation from local macrophages and DCs, and produce large amounts of T cell-associated cytokines [12]. A growing body of literature has identified pleiotropic roles for this novel class of cell in homeostasis and inflammation, as shall be discussed in more depth in later sections.

1.1.3. Adaptive immunity

Adaptive immunity consists of two major lymphocyte populations, B cells and T cells, derived from the common lymphoid progenitor (CLP) in the bone marrow (Fig. 1.1B).

B cells mature in the bone marrow and migrate to the periphery, where their primary function is to develop into antibody-secreting cells – rare terminally-differentiated plasmablasts and plasma cells – upon antigen encounter. All mature B cells express functional antigen-specific B cell receptors (BCRs) that recognise unprocessed antigens and drive B cell activation. These BCRs are identical to the immunoglobulin (Ig) antibody subsequently secreted by the cell. Three classes of mature B cells exist: follicular B cells that reside primarily within the eponymous B cell follicles of secondary lymphoid organs (SLOs), marginal zone (MZ) B cells located in the marginal sinus of the spleen, and B1 cells, enriched in peritoneal and pleural cavities, as well as at mucosal sites [13].

Follicular B cells typically undergo T cell-dependent (TD) responses in response to protein antigens. Primary signalling through the BCR can elicit the generation of short-lived plasmablasts in an extrafollicular responses that results in antibody of limited affinity [14]. Activated B cells can re-enter the B cell follicle and proliferate extensively, under the influence of local T follicular helper (Tfh) cells, to form a germinal centre (GC). Following several rounds of positive selection, GCs ultimately yield high-affinity, long-lived plasma cells that contribute

to sustained high-level antibody production, as well as memory cells [15]. In the presence of T cell-independent (TI) antigens, such as bacterial polysaccharides, plasmablast formation occurs in the absence of GC formation.

T cells are characterised by their expression of the eponymous T cell receptor (TCR), which recognises protein epitopes presented in the context of MHC class I (MHC-I) or MHC class II (MHC-II) on antigen presenting cells (APCs). Two major populations of T cells exist based on their functional characteristics and can be divided based on expression of CD4 and CD8. $CD8^+$ T cells are a cytotoxic subset that kill infected or tumour cells presenting antigen in the context of MHC-I. In contrast, following MHC-II-dependent TCR ligation in SLOs, naïve $CD4^+$ T cells can differentiate into a range of T helper (Th) cells, depending on secondary signals and TCR signal strength. Th subsets differ in their cytokine profile, location, and transcription factor (TF) dependence, and include type 1 (Th1), type 2 (Th2), type 17 (Th17), Tfh, and regulatory (Treg) subsets [16].

Therefore, innate and adaptive immunity exert unique functions that contribute to the protection of the host from invading pathogens and transformed cells. Extensive cross-talk between these two arms is achieved through contact-dependent mechanisms, such as antigen presentation by DCs and engagement of co-stimulatory molecule pairs, as well as through soluble effectors, such as cytokines, and is required for the development of appropriate inflammatory responses. In the following section, focus will switch primarily to the effector functions of the immunoglobulin antibodies produced by activated B cells.

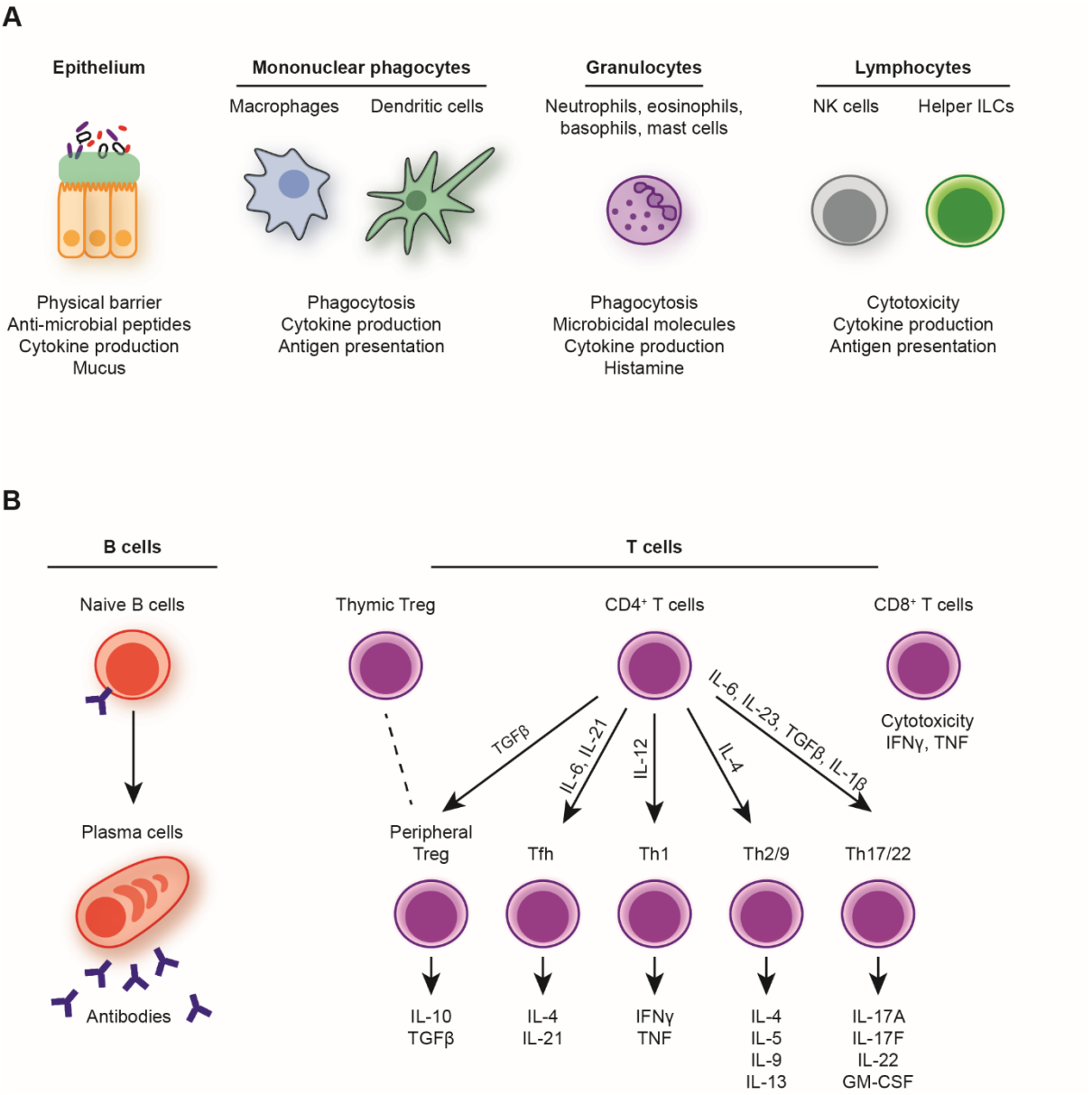

Figure 1.1. Major cellular components of the immune system. Major haematopoietic and non-haematopoietic cells involved in innate (A) and adaptive (B) immune responses.

1.2. Antibodies

1.2.1. Overview

Ig antibodies have taken centre stage in immunological research since the end of the 19th century, when von Behring and Kitasato discovered humoral antitoxins to diphtheria and tetanus. In 1892, von Behring and Wernicke further demonstrated the passive transfer of immunity in animals, with the former receiving the first Nobel Prize in physiology of medicine for these seminal studies and their subsequent use in the treatment of previously intractable diseases. These humoral antitoxins were shown to be directly capable of bacterial lysis, clashing with the cellularist theory of immunity championed by Metchnikoff, which postulated

that phagocytes were responsible to immunity to pathogens [17]. Almonth Wright mediated somewhat this dispute through the discovery of the phenomenon of opsonisation. Opsonins were described as "… what you butter the disease germs with to make your white blood corpuscles eat them.", demonstrating cooperation between cellular and humoral immunity [18].

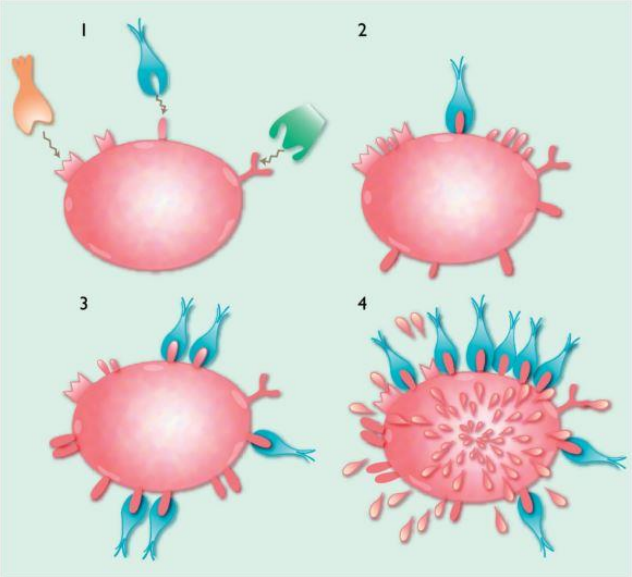

Figure 1.2. Paul Ehrlich's hypothesis of antibody generation. Specific immune cells express receptors that interact with antigens (1). Upon ligation of the receptor (2, 3), cells secrete antibodies resembling the engaged cell surface receptor (4). From Silverstein et al., 2003.

Paul Ehrlich, one of the founding fathers of immunology, conceptualised Ig function into something resembling the modern paradigms of antibody biology, and was the first to coin the term "antibody" (*Antikorper*). He proposed a strictly specific relationship between toxin and antitoxin, with the formation of chemical bonds that eliminate toxin function. Furthermore, he hypothesised the "lock-and-key" model for antibody production: an identical cell receptor, known as a "side-chain", would interact specifically with toxins and induce the production of antibodies/antitoxins (Fig. 1.2) [19]. These theories were subsequently confirmed by the demonstration of the shape-dependent specificity of antibodies by Linus Pauling in the 1940s [20], and the identification of plasma B cells as the source of antibody generation by Astrid Fagraeus in 1947 [21].

We now know antibodies as glycoproteins secreted by plasma cells as part of the humoral immune response, with a diverse range of effector functions (Fig. 1.3). These include their ability to activate complement via the binding and activation of C1q, the activation of immune cells via Fc receptors, and the direct neutralisation of toxins and microbes [22]. Indeed, engagement of distant immune cells through Fc receptors allows for the development of

targeted innate immune responses by cells otherwise lacking somatically-recombined receptors, underlying the observations made by Wright. It is estimated that humans generate approximately 10 billion different antibodies, each capable of binding to distinct antigen epitopes. Their critical importance is starkly exemplified in primary antibody deficiencies, such as X-linked agammaglobulinemia, whereby individuals are susceptible to recurrent bacterial and viral infections, often requiring intravenous immunoglobulin (IVIG) therapy throughout life [23].

Unsurprisingly, given their pleiotropic roles in immunity, detrimental Ig-driven immune responses are associated with several inflammatory and autoimmune disorders, including SLE, Goodpasture's syndrome, and RA. As such, antibodies are a key class of effector molecule that both contribute to anti-microbial immunity, as well as drive susceptibility to autoimmunity, as shall be discussed.

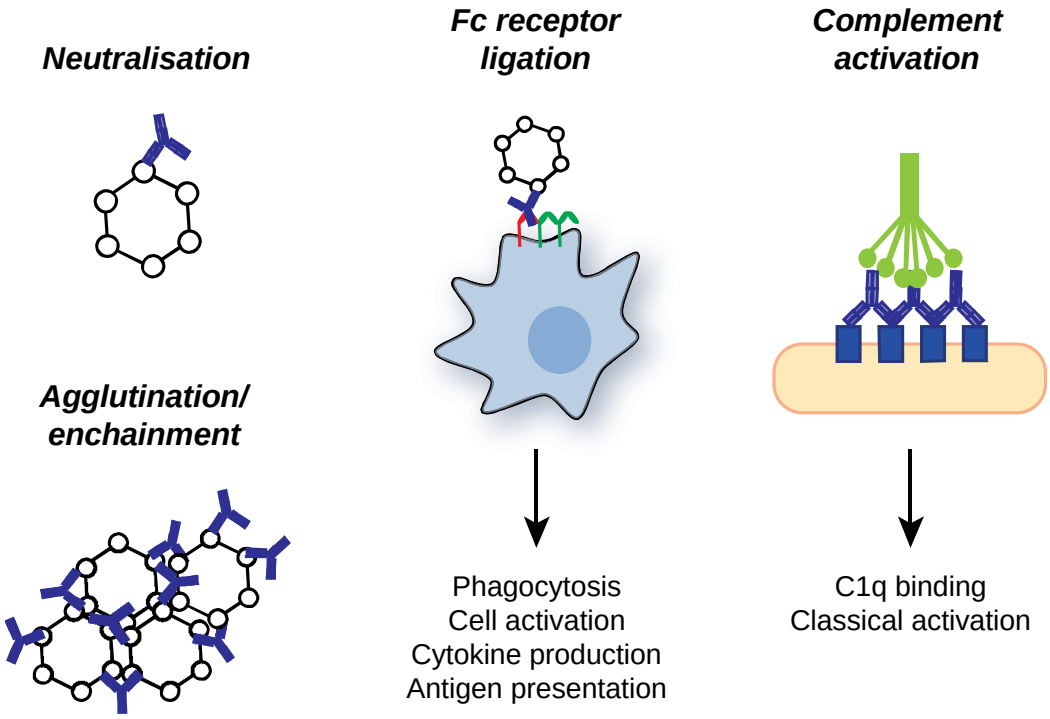

Figure 1.3. Immunoglobulin antibody effector functions. Ig isotypes and subclasses can differentially mediate numerous effector functions, such as toxin/microbe neutralisation and agglutination, Fc receptor engagement, and activation of complement via binding of C1q (green).

1.2.2. Structure

Igs are heterodimeric glycoproteins composed of two heavy (H) and two light (L) chains, with the eponymous Ig domain forming the building block of both chains (Fig. 1.4A). Each chain contains a single variable (V) amino-terminal domain and one, three or four carboxy-terminal constant (C) domains. H chains contain three or four C domains, whereas L chains contain

only one. The high sequence variability of the V domain allows for the generation of antigen binding sites with astounding resolution: discrimination between antigens can occur at the single atom level. Each V domain contains three regions of sequence variability, called complementarity determining regions (CDRs). The three H CDRs and three V CDRs pair to form an unique antigen binding site [24].

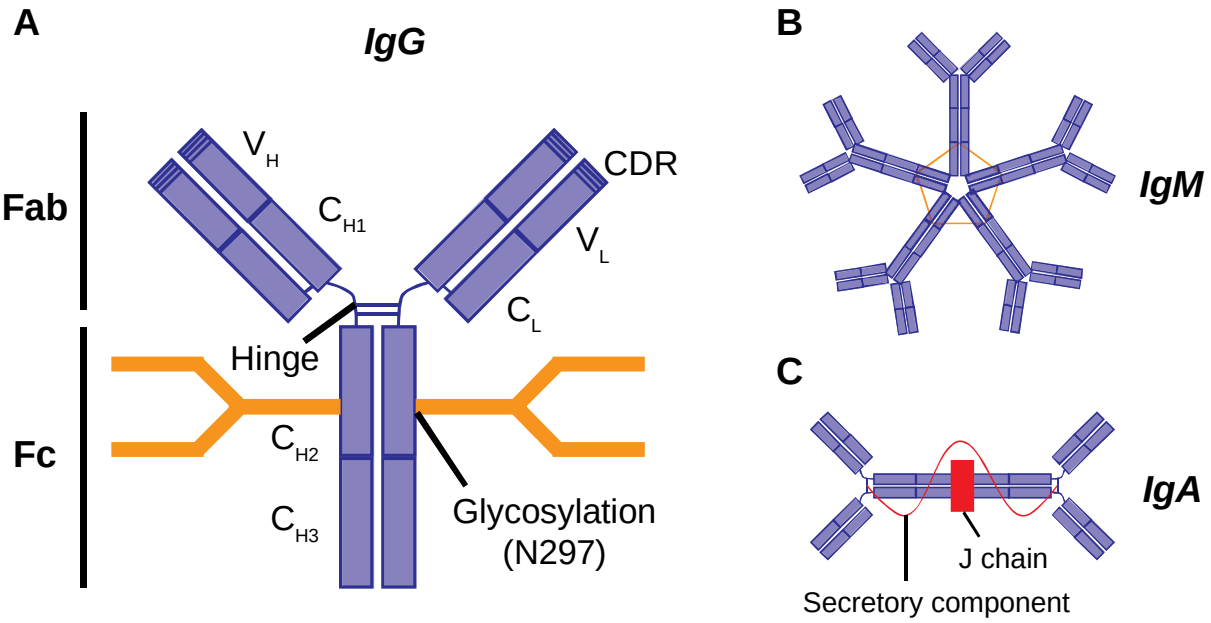

Figure 1.4. Structure of Ig isotypes. (A) Detailed diagram of IgG structure. Overview of oligomeric structure of pentameric IgM (B) and dimeric secretory IgA (C).

Beyond the V domains, the first C domain of the H chain (C_{H1}) associates with the single C domain of the L chain to form a stable platform for V_H and V_L pairing, together forming the Fab domain. The remaining C_{H2} and C_{H3} (for antibodies with a hinge region) or C_{H3} and C_{H4} (for antibodies with an extra C_{H2} domain) form the Fc domain and, together with the hinge region, dictate effector functions of the antibody [25]. The length and flexibility of the hinge region varies extensively among isotypes and subclasses, imparting unique characteristics. The C-terminal domain also encodes a secretory tail that allows the antibodies to exit the cell. Alternatively, the secretory tail is replaced by differential splicing with two membrane/cytoplasmic tail domains, M1 and M2, that control the embedding of Ig into the membrane. This membrane-embedded Ig forms the BCR, the “side-chain” of B cells that dictates their activation.

1.2.3. Isotypes

Antibody isotype is determined by the C_H region, and encodes the effector functions of the antibody. There are five major classes of immunoglobulins in humans, IgM, IgD, IgG, IgA and

IgE, each differing in their antigen specificity, tissue distribution and ability to engage cell surface Fc receptors and complement [25]. IgG and IgA will be the main subjects of this thesis, with a brief introduction to IgM.

1.2.3.1. IgM

IgM is the first antibody to be produced during an immune response, and is also the oldest, found in all vertebrate species. While surface IgM forms the naïve BCR, secretory IgM forms a pentameric complex via disulphide bonds between the C_{H4} domains that allows it to interact avidly with several antigens and bind C1q with 1000-fold greater affinity than IgG (Fig. 1.4B) [24], [26].

Natural polyreactive IgM, found in germ-free (GF) mice, binds to conserved bacterial and host structures, making it particularly effective in promoting the engulfment of apoptotic cells and in early defence against infection. Pertioneal B1 cells are major producers of natural IgM [27], [28]. In contrast, immune IgM, i.e. in response directly to immune challenge, originates from B2 cells and can be both innate-like, TI IgM, such as from MZ B cells [29], and TD, for example in follicular B cells prior to class-switching to other isotypes, as well as MZ B cells.

Due to its large size, IgM cannot enter extravascular spaces, while monomeric IgG, IgE and dimeric IgA can be distributed systemically. Therefore, IgM primarily plays a key role in bridging the temporal gap between antigen exposure and the development of class-switched high-affinity IgA and IgG antibodies for antigen eradication and the generation of memory.

1.2.3.2. IgG

IgG is the major antibody isotype in human serum and extravascular compartments, accounting for 10-20 % of all plasma protein and 70-75 % of total Ig [25]. IgG can be subdivided into four subclasses in humans (IgG1-4), named in order of their relative serum concentration, while four major subclasses exist in mice (IgG1, IgG2a, IgG2b, and IgG3). Despite an estimated 90 % sequence similarity across IgG subclasses, there are differences in structure, function and half-life ascribed to each subclass, as outlined below. Differences in the hinge region appear to be particularly important in this regard. Generally, however, IgG antibodies are known for their high antigen affinity, driven by somatic hypermutation (SHM), and are key molecules involved in immunological memory, serving a variety of inflammatory effector functions, including complement activation via the classical pathway and binding of Fcγ receptors (FcγRs).

IgG1 is the most abundant IgG subclass in humans and is generally associated with soluble protein antigens and membrane proteins. Along with IgG3, IgG1 can potently activate numerous effector mechanisms, including complement activation and FcγR ligation, and is

largely TD [30]. As such, IgG1 is frequently exploited in pharmaceutical settings for the generation of therapeutic monoclonal antibodies [31]. Specific resides on IgG1 C_{H2} are responsible for interaction with C1q, with each IgG having two binding sites for C1q on the Fc domain. Artificial substitution of similar residues in IgG2 is sufficient to impart complement-fixing activity on this subclass [32], [33].

IgG2 responses are almost completely restricted to TI bacterial capsular carbohydrates, although anti-carbohydrate IgG antibodies of other subclasses do exist [34]. IgG2 and IgG4 antibodies have a short rigid hinge region compared to IgG1 and 3, resulting in impaired antibody flexibility, and dictates to a certain extent the affinity of these molecules for FcγRs and C1q. While thought to possess little capacity for complement activation, high epitope densities are associated with IgG2-mediated complement activation [35].

IgG3 antibodies are the most effective subclass in activating effector functions, with enhanced binding to C1q, increased affinity for FcγR binding, and a shorter half-life. This reduced half-life is ascribed to an R435 residue that replaces a histidine in all other subclasses [36]. R435 reduces affinity for the neonatal Fc receptor (FcRn), which normally binds IgG in acidic early endosomes and redirects it away from lysosomal degradation [37], [38]. Furthermore, IgG3 has a very long hinge region and increased molecular flexibility. While a potent pro-inflammatory antibody, IgG3-dominated responses appear to be rare: viral responses can induce IgG1 and IgG3 subclasses, with IgG3 antibodies appearing first during infection.

IgG4 is associated with induction by allergens following repeated or long-term exposure to antigen in a non-infectious setting, as well as in immune responses to parasitic infections [25]. Of interest is the ability of IgG4 to suppress immune responses. Due to a proline-to-serine substitution, studies have shown that IgG4 can spontaneously dissociated into half-molecules, forming novel bispecific IgG antibodies that can no longer cross-link epitopes and potentially exhibit anti-inflammatory characteristics [39]. Furthermore, IgG4 can capture allergens before IgE binding, preventing the activation of mast cells. Finally, IgG4 displays relatively high affinity for the inhibitory Fc receptor, FcγRIIB, resulting in the inhibition of effector cell responses [40]. Therefore, immunotherapy has aimed at driving IgG1/3/E-dominated immune responses towards IgG4 as a means to dampen autoimmunity and inflammation [41].

1.2.3.3. IgA

While only constituting 10-15 % of total serum Ig, IgA is the major Ig isotype at mucosal surfaces. Secretory IgA (S-IgA) consists of IgA dimers covalently linked by the joining (J) chain and one secretory component (SC) chain, the extracellular portion of the polymeric Ig receptor (pIgR) (Fig. 1.4C). pIgR is expressed in mucosal epithelial cells and transfers polymeric IgA or IgM from basolateral to apical surfaces [42].

Two IgA subclasses exist in humans: IgA1 comprises 80 % of IgA in serum, while IgA2 is the major form in certain mucosal secretions, including in the large intestinal (LI) mucosa, the latter thought to be driven by the enhanced resistance of IgA2 to proteolysis resulting from its shorter hinge region.

While considered to be a largely non-inflammatory isotype, given the relative scarcity of Fcα receptors and an impaired ability to activate complement, S-IgA participates in shaping the mucosal microbiota, immune maturation, and the resistance to pathogens through several mechanisms, collectively referred to as immune exclusion. This will be discussed in more detail in subsequent sections.

1.2.4. Class-switch recombination

The emergence of specific Ig isotypes is tightly regulated to suit the requirements of the immune response. Following BCR activation, Ig isotype switching occurs by an intra-chromosomal deletion recombination event known as CSR, the replacement of the default expressed C_μ exon cluster in naïve B cells with Cγ, Cϵ, or Cα, for IgG, IgE, and IgA, respectively [43]. The regulation of CSR, rather than the mechanism itself, will be discussed here.

1.2.4.1. Primary signals

Primary signals in CSR are involved in the induction of activation-induced cytidine deaminase (AID) and other CSR factors [43]. B cell CD40 engagement by CD40L found on Tfh cells occurs within GCs and is essential for the induction of AID [44]. Other than CD40L, Tfh cells also express co-stimulatory molecules, such as PD-1 and OX40, and the B cell-activating cytokines interleukin (IL)-4 and IL-21 [45]. As a consequence, GCs, memory and plasma B cell formation, and Ig production are severely impaired in the absence of Tfh cells or CD40 ligation [46].

In the absence of CD40 ligation, B cell-activating factor (BAFF) and a proliferation-inducing ligand (APRIL) can provide a primary signal in TI class-switching by extrafollicular B cells [47]. These tumour necrosis factor (TNF) ligand superfamily members engage BAFF receptor, B cell maturation antigen (BCMA), and transmembrane activator and CAML interactor (TACI) to stimulate AID expression [48]. BAFF and APRIL are released in response to Toll-like receptor (TLR) stimulation by several cell subsets, including neutrophils, eosinophils, ILCs, DCs, follicular dendritic cells (FDCs), and epithelial cells [47], [49]–[53].

Murine IgG3 levels are relatively unaffected by the absence of CD40 or T cells [54]. TLR signalling is involved in polysaccharide-specific IgG generation and immune responses to encapsulated bacteria. TLR1-2, TLR4, TLR7 and TLR8 can synergise with the BCR to induce class-switching to IgG3 and other isotypes in the absence of T cell help [48], [54], [55].

Lipopolysaccharide (LPS) found in the outer membrane of Gram-negative bacteria is the only known microbial product that can directly induce CSR through simultaneous TLR4 ligation and BCR cross-linking in murine B cells. It should be noted that BCR activation is not sufficient to induce AID expression, and likely reflects a regulatory checkpoint prior to class-switching.

1.2.4.2. Secondary signals

Secondary T cell- and DC-derived cytokines determine the fate of CSR by regulating transcription from the S regions, short tandem repeats located upstream of each C_H region. IL-4 supports class-switching to IgG1 and IgE in the presence of CD40 ligation via signal transducer and activator of transcription (STAT)-6, while IFNγ and transforming growth factor beta 1 (TGFβ1) support class-switching to IgG2a and IgA via SMAD3 and SMAD4 or Runt-related transcription factor (RUNX) proteins, respectively [56], [57]. Indeed, IFNγ secretion by $CD8^+$ T cells supports murine IgG2a/b production *in vivo* [58]. Furthermore, recent data has demonstrated that Th17 cells support IgG generation *in vivo*. While IL-17A promoted IgG2a and IgG3, IL-21 drove class-switching to IgG2b and IgG1 class-switching in mice [59]. IL-21 secretion by Tfh cells is also essential for antibody responses by stimulating B cell proliferation and expression of *AICDA*, *BLIMP1*, and *XBP1* [60].

Recently, Tfh cells were found to progressively secrete IL-21 and IL-4 for the induction of high-affinity BCR clones and the development of Blimp-1-dependent plasma cells, respectively [61]. Therefore, Tfh cells can provide both primary and secondary signals over the course of the GC reaction.

In summary, efficient TD antibody responses require primary CD40 ligation by Tfh cells and secondary cytokine signals and dictate CSR. However, in the absence of T cells, TI antibody production can arise following BAFF/APRIL- or TLR-mediated primary signals that induce the expression of AID.

1.3. Fcγ receptors

1.3.1. Overview

FcγRs are cell surface glycoproteins that bind to the Fc portion of IgG antibodies [62]. They are responsible for mediating numerous cellular effects of IgG, including immune cell maturation and migration, the production of inflammatory mediators, and the elimination of opsonised microbes.

There are several activating FcγRs and a single inhibitory receptor, FcγRIIB, in both mice and humans (Fig. 1.5). Other than FcγRI (and FcγRIV in mice), most FcγRs are low-to-medium affinity for IgG, requiring cross-linking of several receptors into signalling synapses on the cell surface to initiate productive signalling. This is achieved through the formation of high avidity

antigen:antibody immune complexes (IC), aggregated IgG, or by IgG-opsonised cells and surfaces. The absence of signalling upon ligation of monomeric IgG prevents inappropriate immune cell activation, which is critical given the abundance of circulating monomeric IgG. The inhibitory receptor, FcγRIIB, acts as an additional regulatory mechanism to suppress IgG-mediated inflammation [62], [63].

As well as IgG, certain murine FcγRs (FcγRIIB, FcγRIII, and FcγRIV) are able to bind IgE. While IgE affinity is approximately 2 log lower, this should be borne in mind when ascribing Ig function [64].

1.3.2. Signalling

Crosslinking of activating FcγRs results in the phosphorylation of immunoreceptor tyrosine-based activating motifs (ITAMs) located on the intracellular domain of FcγRs or on the associated common γ-chain (also known as FcεRIγ/FcRγ) [65], [66]. ITAM phosphorylation activates signalling cascades via SRC family kinases and spleen tyrosine kinase (SYK), resulting in the downstream activation of phosphatidylinositol3-kinase (PI3-K) and phospholipase-Cγ. This subsequently triggers protein kinase C and a calcium flux [62], [64], [66]. Importantly, binding of monomeric IgG to high affinity FcγRs has not been shown to induce activating signalling.

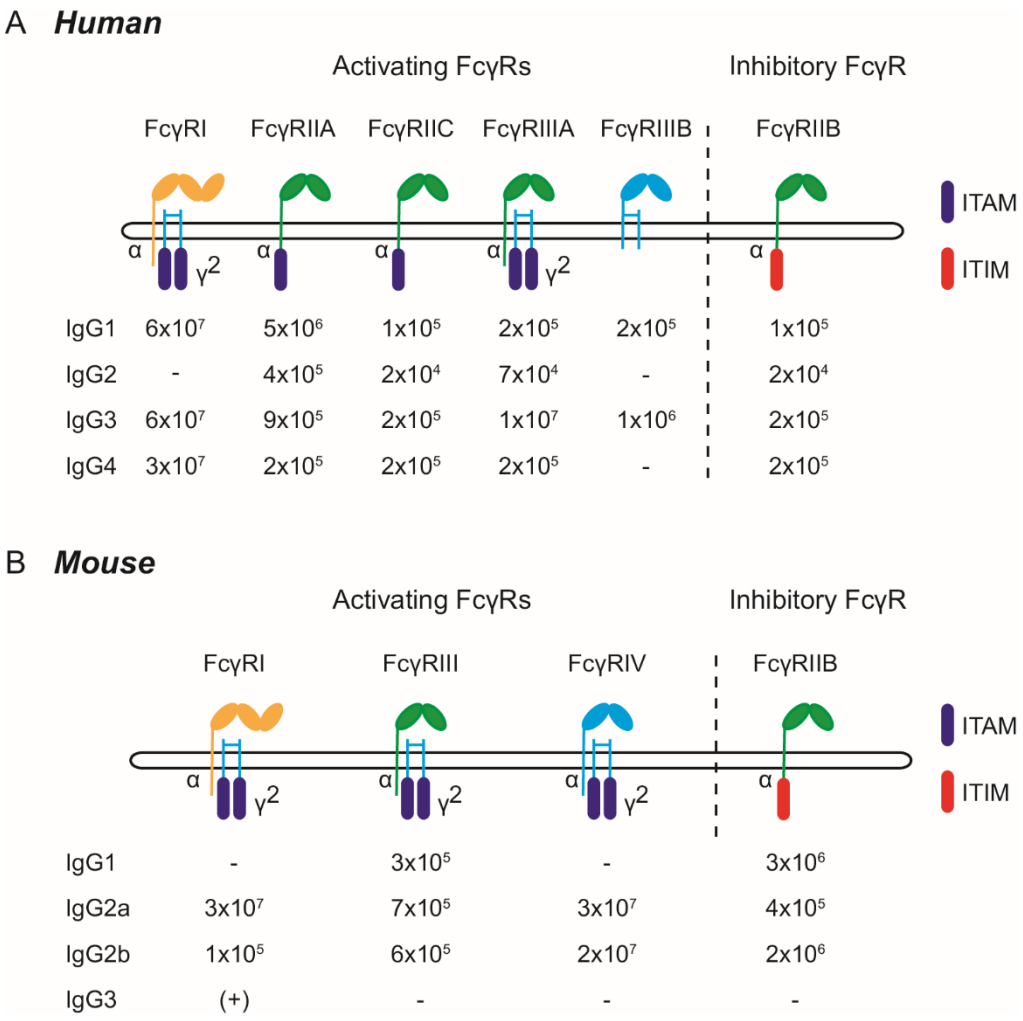

Figure 1.5. Human and murine Fcγ receptors and affinity for IgG subclasses. (A) Human FcγRs and affinity to IgG subclasses (K_A (M^{-1})), divided into activating (left) and inhibitory (right) receptors. (B) Murine FcγRs depicted in the same way. γ^2 = common γ-chain. Adapted from Bruhns & Jonsson, 2015.

The inhibitory FcγRIIB contains an intracellular immunoreceptor tyrosine-based inhibitory motif (ITIM). Cross-linking of FcγRIIB with activating FcγRs leads to ITIM phosphorylation by SRC kinases and the recruitment of inositol phosphatases, most notably phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1 (SHIP1). These phosphatases act to neutralise activating signals and dampen IgG-mediated responses [62], [67]. Unsurprisingly, therefore, activating and inhibitory FcγRs are co-expressed on many immune cells, with their relative expression level and activity setting the cellular activation threshold upon encounter of ICs and opsonised cells.

1.3.3. Functions

FcγRs are widely expressed on immune cells, including neutrophils, monocytes, macrophages, DCs, mast cells, NK cells and B cells. However, the FcγR repertoire differs

between immune cells. Furthermore, the immune cell phenotype dictates the effect of IgG-mediated FcγR cross-linking, as shall be discussed. These effects are summarised in Figure 1.6.

1.3.3.1. Macrophages

Both *ex vivo*-isolated and *in vitro*-generated macrophages express elevated levels of both activating and inhibitory FcγRs, with the ratio skewed in favour of activating signalling. Indeed, FcγRI (CD64) is often used as a specific marker to discriminate between tissue-resident macrophages ($FcγRI^+$) and DCs ($FcγRI^-$) [68]. As well as FcγRI, human and murine macrophages express the full complement of canonical FcγRs, except for FcγRIIIB in humans.

The role of FcγRs in macrophage activation is widespread and has recently been excellently reviewed [69], [70]. Internalisation of opsonised material or ICs is a common function shared among all FcγRs, although the fate of internalised cargo differs depending on the class of Fc receptor. ITAM-bearing activating receptors favour a degradative route for antigen processing and presentation. In contrast, the ITIM-bearing FcγRIIB has been proposed to favour a non-degradative route of internalisation for transfer of intact antigen to B cells [69], [71].

FcγRs also regulate the production of inflammatory mediators by macrophages. Historically, macrophages can be classified as so-called M1 and M2 macrophages [72]. IFNγ-activated M1 macrophages are proposed to represent inflammatory tissue macrophages that produce IL-12, IL-1, and TNF in response to LPS. In contrast, M2 macrophages are generated *in vitro* through IL-4 stimulation and are characterised by production of anti-inflammatory molecules, such as arginase and IL-10. *In vitro* studies with human monocyte-derived macrophages (MDMs) and murine bone marrow-derived macrophages (BMDMs) polarised in this way have allowed the elucidation of FcγR-induced activation.

FcγR-mediated human monocyte and macrophage activation induces pro-inflammatory cytokine production characterised by production of IL-1β, IL-6, IL-10, IL-12 and TNF-α, as well as chemokines including CXCL8 [73]. Interestingly, several studies have highlighted a link between TLR and FcγR co-stimulation and the induction of a Th17-inducible macrophage phenotype. Co-stimulation of human M2 macrophages with IgG-opsonised bacteria resulted in potent production of IL-1β [74]. A similar phenotype is also seen in human monocyte-derived DCs (MDDCs) [75], [76], while IgG opsonisation of *Plasmodium falciparum*-infected erythrocytes increased inflammasome-dependent IL-1β production by human macrophages [77], [78]. Strikingly, M1-polarised MDMs did not respond in this way, suggesting that FcγR-mediated Th17 induction is a feature unique to “anti-inflammatory” macrophages. In murine BMDMs, LPS and IC co-stimulation results in the inhibition of LPS-induced IL-1β and TNF production, at least partly dependent on production of prostaglandin E2 (PGE2) [79], [80].

While helpful in dissecting the mechanistic roles of FcγR signalling, the *in vitro*-defined subsets of macrophages, in many circumstances, poorly mimic the complex macrophage phenotypes observed *in vivo*. For example, macrophages induced following mycobacterial infection resemble a mixed M1/M2 phenotype, with high levels of IL-1β, TNFα, IL-10, and IL-12 [81]. Strikingly, these cells resemble IC-elicited M2 macrophages and could similarly skew T cell responses towards IL-17A and IL-22 production. Similarly, intestinal macrophages are characterised by high levels of IL-10 and TNFα, and their reprogramming to potent IL-1β-producing cells during intestinal inflammation that can support Th17 differentiation [82], [83].

1.3.3.2. Dendritic cells

An essential function of DCs is the transportation of antigen via the CCR7-CCL19/21 axis from tissues to local draining lymph nodes for antigen presentation to T cells.

While DCs express FcγRI and FcγRIIA in humans, and FcγRIII in mice, FcγR expression is skewed towards FcγRIIB in immature DCs. DC FcγR expression is tightly regulated, with maturation signals, such as LPS or IFN-γ, down-regulating FcγRIIB to allow for IgG-induced cell activation via activating FcγR ligation [84]. DCs efficiently process FcγR-internalised antigen and upregulate MHC and co-stimulatory molecules for robust antigen presentation to T cells [85]–[87]. A recent study demonstrated that FcγR cross-linking induces CCR7 and matrix metalloprotease (MMP) expression that facilitates DC migration from inflamed peripheral sites to local draining lymph nodes [88]. Therefore, FcγRs play a critical role in antigen presentation to T cells through a variety of mechanisms.

As previously mentioned, studies have demonstrated cytokine and chemokine production by DCs in response to FcγR signalling, both directly and through synergism with TLR signalling [75], [76]. FcγRIIB blockade leads to spontaneous DC maturation and the induction of a cytokine programme characterised by TNF-α, IL-6, CXCL8, and IL-12p70 production, similarly to macrophages [73], [84], [89].

1.3.3.3. Neutrophils

Human neutrophils constitutively express FcγRIIA, FcγRIIC and FcγRIIIB, as well as low levels of FcγRIIB, which is relative refractory to *in vitro* cell stimulation [90], [91]. Murine neutrophils predominantly express FcγRIII and FcγRIV, with similarly low levels of FcγRIIB expression at rest [92], [93]. Cross-linking of activating FcγRs has profound effects on neutrophil function, as one might expect of cells poised to acutely respond to a variety of stresses. FcγR-inducible mechanisms include intracellular calcium fluxing [94], antigen phagocytosis [95], [96], the generation of a respiratory burst [97], neutrophil adhesion to endothelial cells [98], neutrophil extracellular trap formation [99], [100], and cytokine production, including TNF-α, IL-1β and oncostatin M (OSM) [96], [101].

1.3.3.4. Natural killer cells

NK cells express activating FcγRIIC and FcγRIIIA in humans, and FcγRIII in mice, but not FcγRIIB [102], [103]. Indeed, CD16 (FcγRIII) is often used to discriminate between cytotoxic $CD56^{dim}$ NK cells ($CD16^{+}$) and $CD56^{bright}$ NK cells ($CD16^{-}$). FcγR signalling on NK cells stimulates the targeted release of cytotoxic molecules to kill opsonised cells, a process known as antibody-dependent cell-mediated cytotoxicity (ADCC). As well as ADCC, NK cells undergo IFN-γ and TNF-α release following FcγR cross-linking [104], [105].

1.3.3.5. B cells

FcγRIIB is the only FcγR expressed by B cells, where it cross-links to the BCR to increase the cellular activation threshold and suppress antibody production [62]. Furthermore, direct cross-linking of FcγRIIB on the surface of mature B cells and bone marrow-resident plasma cells can directly mediate apoptosis, thereby limiting the peripheral pool of antibody-producing cells [106], [107]. As such, FcγRIIB has a critical role in maintaining humoral tolerance.

1.3.3.6. Non-haematopoietic cells

In addition to the well-described roles for FcγR signalling on immune cells, evidence suggests that non-haematopoietic cells can also undergo cellular activation in response to IgG binding.

FDCs are stromal cells located within B cell follicles in SLOs [108]. FcγRIIB is highly expressed on GC FDCs and involved in immune complexed antigen capture, with impaired antibody and memory B-cell responses in its absence [109], [110].

A significant body of literature has demonstrated effects of IgG on endothelial cells, both through FcγR-dependent and independent mechanisms. In antibody-mediated rejection (ABMR) following transplantation, donor-specific IgG antibodies (DSAs) can directly mediate endothelial cell activation via binding to surface MHC [111]–[113], while FcγRs on cultured human aortic endothelial cells were shown to induce phagocytosis, cytokine production, and the upregulation of adhesion molecules in response to C-reactive protein (CRP) [114]. TNF-α and IFNγ have been shown to further enhance FcγR expression by human endothelial cells *in vitro*, and this may have added importance during tissue inflammation [115]. Finally, FcγRIIB was implicated in protection against obesity-induced hypertension through IgG-mediated attenuation of endothelial nitric oxide (NO) synthase activity in mice [116].

Therefore, activating FcγRs are widely expressed on cells of the innate immune system, as well as B cells and non-haematopoietic cells, where they mediate cell maturation, antigen presentation, cytokine production, and target-cell destruction in response to IgG ligation. Furthermore, intrinsic inhibitory FcγRIIB plays essential roles in the suppression of these effects.

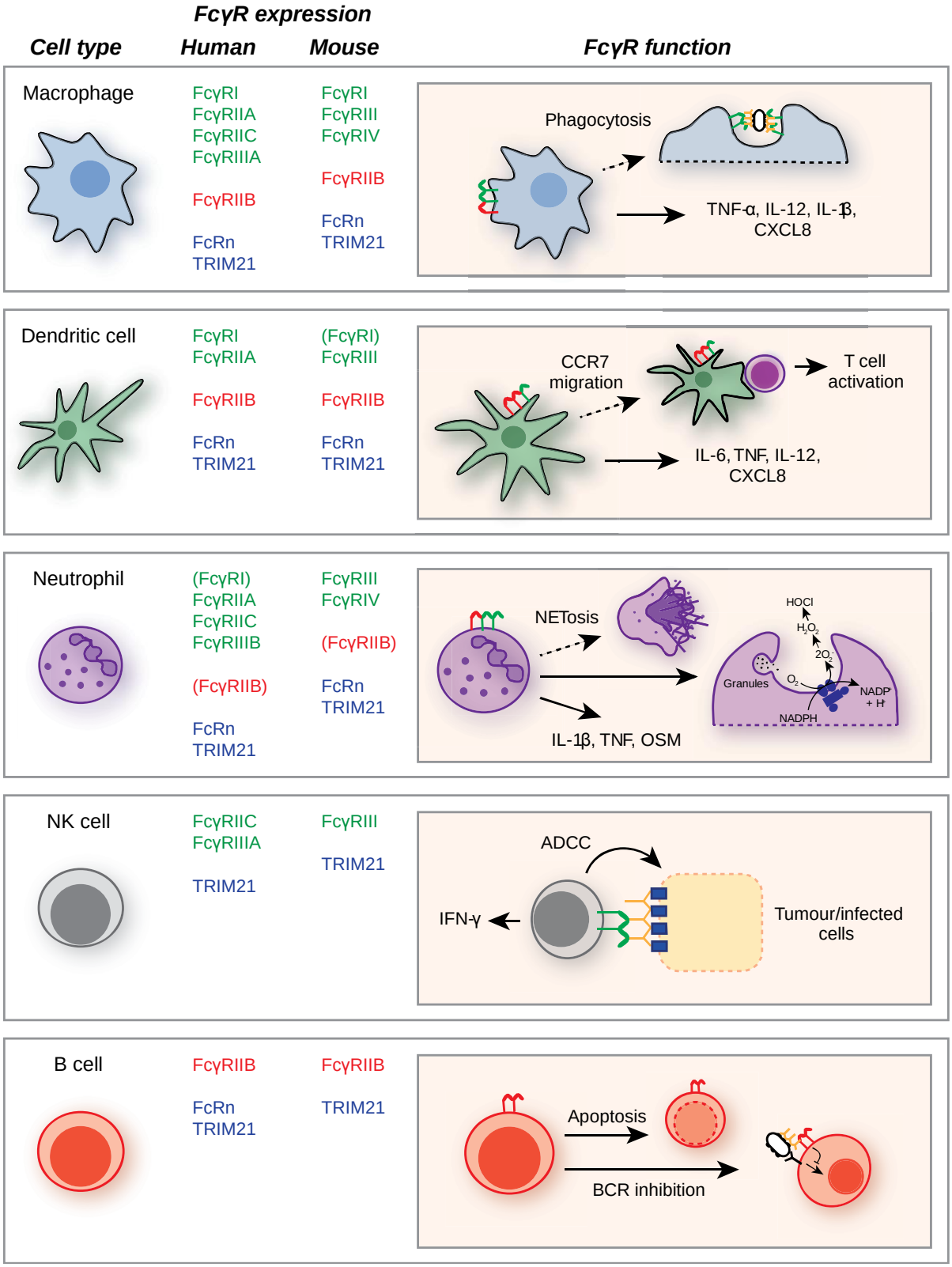

Figure 1.6. Effect of FcγR cross-linking on major immune cell populations. FcγR expression across cell types is depicted. Green = canonical activating FcγRs; red = canonical inhibitory FcγR; blue = non-canonical FcγRs.

1.3.4. Innate signals and FcγRs

FcγRs not only serve to bind IgG, but can also signal in response to ligation by other soluble effectors. Furthermore, FcγRIIB can influence non-FcγR signalling pathways, such as TLRs, as shall be discussed (Fig. 1.7).

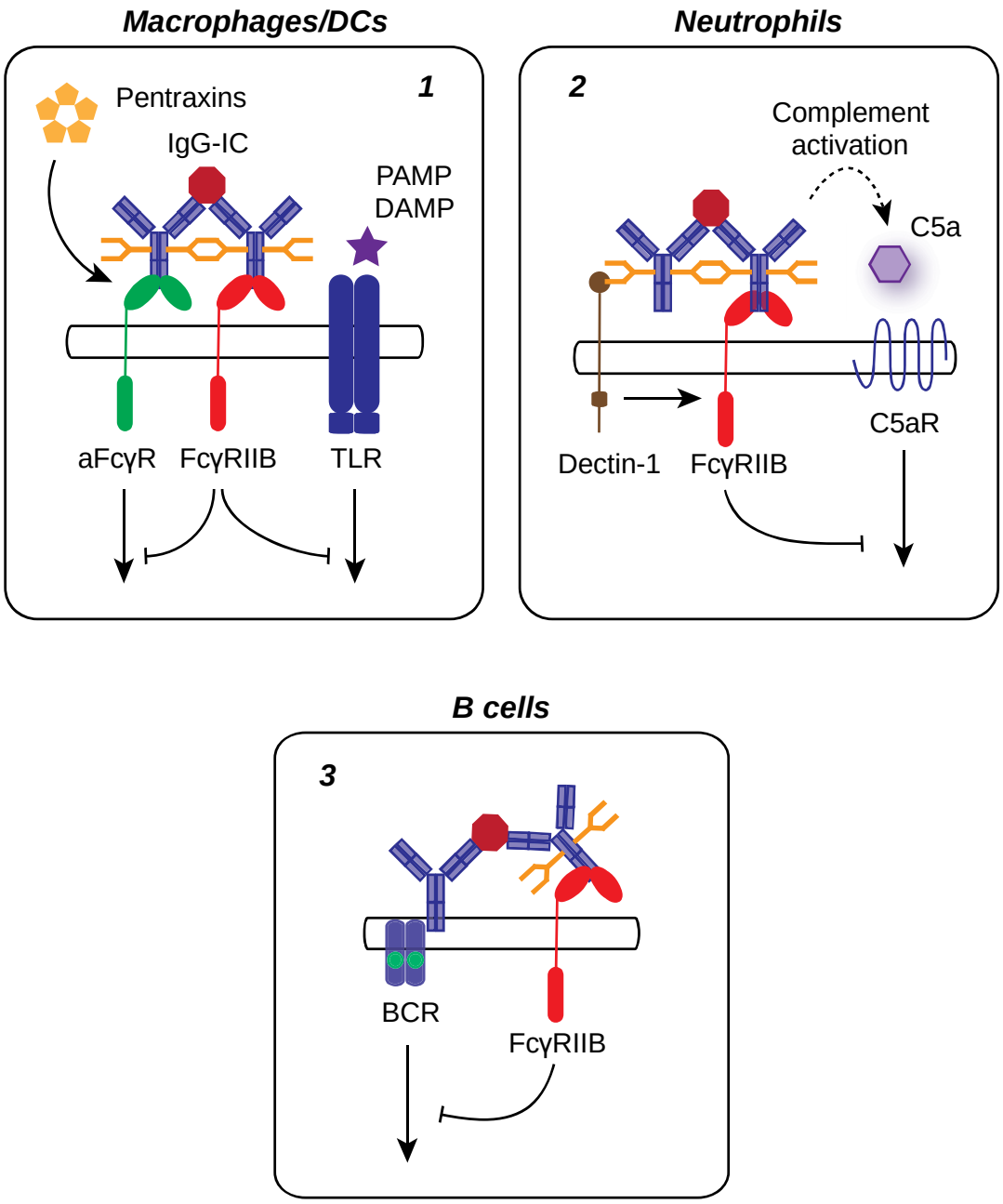

Figure 1.7. Canonical and non-canonical functions of FcγRs. Activating FcγRs can bind both IgG and pentraxins, both inhibited by FcγRIIB (panel 1). FcγRIIB can cross-link to TLRs (panel 1), complement receptors and C-type lectin receptors (panel 2). FcγRIIB is known to inhibit signalling from the BCR by SHIP1-mediated dephosphorylation of BCR-associated ITAMs (panel 3). Adapted from Espeli, Smith & Clatworthy, 2016.

1.3.4.1. Pentraxins

FcγRs can bind to pentraxins, including CRP and serum amyloid P (SAP), demonstrating a link between FcγRs and innate humoral immunity [117], [118]. SAP was shown to bind to FcγRI, IIA, and IIIB on human polymorphonuclear leukocytes (PMNs), while CRP predominantly binds to FcγRIIA on human monocytes and neutrophils. CRP, a commonly used biomarker in inflammatory disease, binds to phosphorylcholine moieties on microbes and necrotic cells, as well as chromatin and histones, allowing for FcγR-mediated internalisation and disposal of damaged cells and self-antigens, while also potentially activating inflammatory responses in FcγR-expressing cells [63], [119], [120].

1.3.4.2. FcγRIIB and innate immune receptors

FcγRIIB can inhibit activating signals from non-FcγR cell-surface receptors via the recruitment of SHIP1 to signalling synapses. This so-called *trans-inhibition* was demonstrated for Kit-dependent mast cell activation [121], [122], and TLR4 and Ca5R signalling in innate cells [79], [123]. In the latter, this was in part dependent on the association of FcγRIIB with dectin-1 mediated by IgG galactosylation.

1.3.5. Modulating FcγR function

The regulation of FcγR expression by immune cells influences their ability to respond to IgG-ICs. The so-called activating-to-inhibitory (A/I) ratio, i.e. the relative expression or activity of activating to inhibitory receptors, has important implications for immune cell activation in the context of an inflammatory response, as well as in tissue-specific immunity, whereby the unique homeostatic milieu can regulate cellular IgG responsiveness [124]. Furthermore, differences in the IgG glycome, FcγR gene polymorphisms, and gene copy number variation (CNV) can also influence the A/I ratio, with implications for inflammatory disease (Fig. 1.8).

1.3.5.1. Expression

IFNγ is associated with an increase in the A/I ratio of several cells: cultured monocytes and MDDCs downregulate expression of FcγRIIB is response to IFNγ [84], as well as upregulating activating FcγRIIA expression [115], [125]. PGE2 also upregulated FcγRIIA expression on MDDCs *in vitro* [84]. In contrast, Th2 cytokines, such as IL-4, have the opposite effect, upregulating FcγRIIB and downregulating activating FcγR expression, to decrease monocyte A/I ratios [125].

1.3.5.2. Glycosylation

Glycosylation is a post-translational modification that alters the structure and activity of glycoproteins. Each IgG heavy chain carries a single covalently attached biantennary *N*-

glycan at the highly conserved asparagine 297 residue in each of the Fc fragment Cγ2 domains, with over 900 IgG glycoforms possible [126]. Biantennary complexes can contain additional bisecting *N*-acetylglucosamine (GlcNAc), core fucose, galactose and sialic acid residues [127]. IgG glycosylation is required for IgG stability and effector function [128], with differences in IgG glycosylation altering affinity for activating versus inhibitory FcγRs and, therefore, modulating the strength of FcγR signalling within cells [129]–[132].

The presence of fucose on core glycans alters binding to FcγRIIIA, with defucosylation resulting in increased binding affinity through increased Fc interaction with the Asn-162 glycan on the receptor [133]. Agalactosylated IgG, with two oligosaccharide chains ending in GlcNAc rather than galactose/sialic acid, is termed G0 (no galactose) [63]. G0 IgG can activate complement via binding to mannose-binding lectin, and can bind the mannose receptor on phagocytes [134], [135]. In contrast, sialylation promotes anti-inflammatory functions of IgG by reducing FcγR affinity and promoting binding to the C-type lectin receptor, SIGN-R1 (DC-SIGN in humans) [136]–[138]. Indeed, sialylation is required for the protective functions of IVIG. Furthermore, binding to C-type lectin receptors causes structural changes in IgG that reduce binding to classical FcγRs [139].

1.3.5.3. Single nucleotide polymorphisms

Single nucleotide polymorphisms (SNPs) are found in *FCGR* genes that alter receptor expression and function, while CNV also determines the levels of FcγR expression on different cell types.

FCGR2A - A polymorphism in FcγRIIA encoding a histidine or arginine at position 131 (H/R131) within the second Ig-like domain results in altered ligand affinity [140], [141]. H131 results in higher affinity for IgG1 and IgG2 compared to R131, with little change in affinity for IgG3 and IgG4 [30], [142]. Furthermore, a splice variant of FcγRIIA (FcγRIIA-exon 6*) is associated with increased neutrophil response to IgG [143].

FCGR2B - SNPs in FcγRIIB are well-studied, with one SNP in human *FCGR2B* gene (FcγRIIB^{T232}; rs1050501) found at high frequency in the population. This polymorphism encodes an isoleucine-to-threonine substitution in the transmembrane domain that results in receptor loss-of-function, mediated by impaired lateral mobility and receptor exclusion from lipid rafts [144]–[146]. Two further SNPs (386(G/c) and 120(T/a)) collectively constitute the 2B.4 promoter haplotype. The c/a haplotype results in increased promoter activity and FcγRIIB expression on a variety of immune cells [62], [147].

FCGR2C – A glutamine at position 13 (Q13) allows for functional receptor expression in 20 % of individuals. In the remaining 80 %, a stop codon is present (stop13) and *FCGR2C*

represents a pseudogene. As a result, NK cells expressing FcγRIIC exhibit enhanced IgG-mediated cytotoxicity [102].

FCGR3A – The FcγRIIIA-V158 variant, encoding a valine rather than a phenylalanine at position 158, exhibits enhanced affinity for all IgG subclasses [30]. CNV is associated with efficacy of NK-mediated cytotoxicity [148].

FCGR3B – Three variants of FcγRIIIB exist: NA1, NA2 and SH. However, differences in IgG binding affinity were not detected across variants [30]. NA1 is associated with enhanced neutrophil uptake of IgG-opsonised erythrocytes [149], while SH enhances FcγRIIIB surface expression [150]. CNV of *FCGR3B* is also correlated with cell-surface receptor expression levels, IC uptake, and soluble serum FcγRIIIB levels [91].

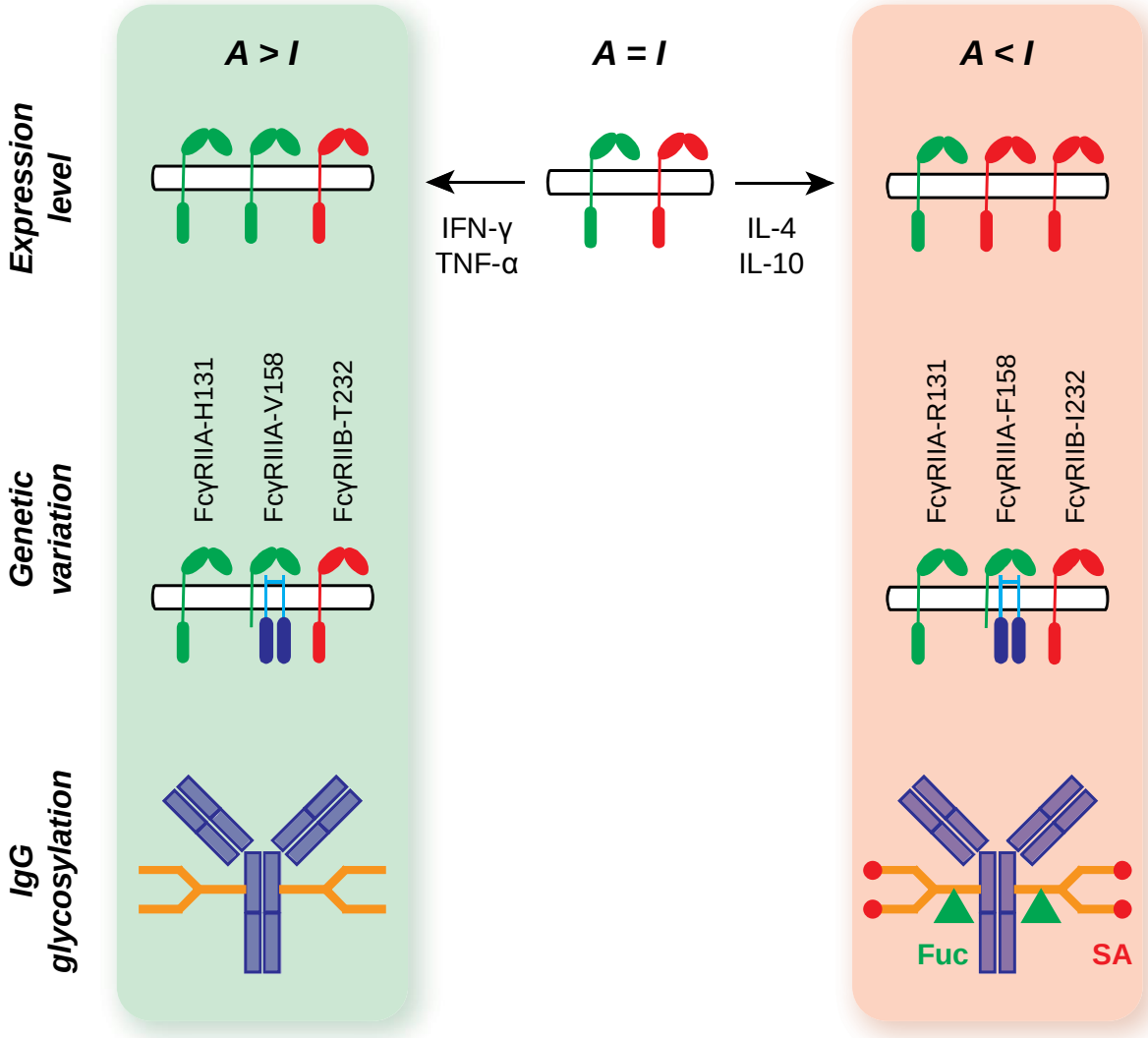

Figure 1.8. Modulation of FcγR activating-to-inhibitory ratio. FcγR signalling can be modulated by cytokine signals that alter cellular FcγR expression, genetic variants that alter receptor expression and/or function, and glycosylation of IgG, a modification that alters FcγR-binding capacity of IgG. SA = Sialic acid; fuc = fucose. Adapted from Espeli, Smith & Clatworthy, 2016.

1.4. Fcγ receptors and disease

Given their roles in mediating numerous inflammatory mechanisms *in vivo*, it is unsurprising that FcγR-mediated signalling and immune cell activation is associated with several autoimmune disorders associated with IgG, as well as in differential susceptibility to infection.

1.4.1. *FCGR* SNPs in autoimmunity and infection

Genetic polymorphisms in human *FCGR* genes are frequently associated with differential susceptibility to infection, autoimmunity, and monoclonal antibody responses [62], [144], [151]. However, these associations are complex. Increased IgG affinity can promote detrimental autoimmune progression, while promoting responses to therapeutic antibodies. In contrast, reduced IgG affinity is associated with impaired IC clearance, which is a risk factor for SLE [152].

FCGR2A – The high-affinity H131 polymorphism is associated with enhanced detrimental immunity in patients with Kawasaki disease, systemic vasculitis treated with IVIG [153], and IBD [154]–[156]. Furthermore, H131 increases response to rituximab (anti-CD20 IgG) therapy in B-cell lymphomas [157], while the low-affinity R131 variant is associated with susceptibility to SLE and graft rejection [158]–[160].

FCGR2B – FcγRIIB-T232, encoding the dysfunctional inhibitory receptor, is associated with susceptibility to SLE, an autoimmune disorder characterised by IgG deposition within different organs, particularly the kidney [161], [162]. Indeed, macrophages and DCs isolated from T/T232 homozygous individuals exhibit an exacerbated pro-inflammatory response to IgG-IC [163]. Conversely, however, FcγRIIB dysfunction is associated with enhanced protection against infectious diseases in humans, with complementary demonstrations in mice. In African and South East Asian populations, FcγRIIB-T232 homozygosity can exceed 10 % and is associated with protection against malaria (odds ratio (OR) = 0.56; $P = 7.1 \times 10^{-5}$) [162]. Curiously, the 2B.4 promoter haplotype is also overrepresented in SLE, implicating FcγRIIB in pathogenesis [147].

FCGR2C – FcγRIIC expression and CNV are associated with Kawasaki disease, consistent with detrimental high-affinity *FCGR2A* SNP associations with this disease [164].

FCGR3A – The V158 variant, with enhanced IgG affinity, is associated with susceptibility to rheumatoid arthritis [165], [166] and immune-mediated thrombocytopenic purpura (ITP) – a disorder characterised by platelet destruction [167] – but a better clinical response to rituximab in B-cell lymphomas [157], [168], a lower risk of recurrent periodontitis [169], and improved outcome in bone marrow transplantation [170]. Furthermore, CNV is associated with anti-glomerular basement membrane disease [171]. In contrast, the low-affinity F158 variant is associated with SLE, consistent with impaired IC clearance [152].

FCGR3B – Low FcγRIIIB expression, as a result of reduced CNV, and the FcγRIIIB-NA2 variant are associated with susceptibility to SLE, consistent with a role for FcγRs in IC clearance [91], [172], [173].

It is clear, therefore, that polymorphisms affecting FcγR expression, IgG affinity, and function has wide-ranging consequences in human disease and responses to therapeutic intervention.

1.4.2. Glycosylation in disease

Abnormalities in the IgG glycome have been identified in patients several autoimmune disorders, as well as in individuals with impaired responses to infectious agents, such as *Mycobacterium tuberculosis.* Agalactosylation is observed in patients with RA, as well as in SLE, which may favour binding to activating FcγRs, while reductions in sialylation are also observed in SLE [126], [174], [175]. A reduction in IgG galactosylation has also been observed in IBD, while decreased IgG sialylation was detected in Crohn's disease (CD), specifically [127], [176].

Seminal work by Alter and colleagues has allowed large-scale profiling of IgG glycomes in disease [78], [177]. Via a "systems serology" approach, they have demonstrated distinct IgG glycosylation patterns, correlated with IgG functionality and immune reactivity, in patients with *M. tuberculosis* and in human immunodeficiency virus (HIV) vaccine trails. Specifically, successful pathogen management was associated with reduced fucose, G0, and increased sialic acid, resulting in enhanced FcγRIIA binding, ADCC, and inflammasome activation.

In further support of the contribution of altered glycosylation patterns in disease development, SNPs in glycosyltransferases are associated with several autoimmune disorders [126]. The induction of these genes is dynamically regulated by the immune system. An elegant recent study demonstrated that in response to IL-23, Th17 cells produced IL-22 and IL-21 to alter the glycosylation profile of B cells in a murine model of RA. Specifically, IL-23 was required for the desialylation of IgG, mirroring RA-associated glycosylation patterns, and augmented inflammatory FcγR signalling by macrophages [178]. Therefore, as well as IgG class-switching, Th17 cytokines can also inflammatory profile of IgG produced by B cells.

1.4.3. Murine models

Murine transgenic models have been essential for elucidating the role of FcγR signalling *in vivo* during homeostasis and inflammatory responses, with several transgenic strains available, from single- and pan-FcγR knockout (KO) mice, lines with transgenic FcγR overexpression, and humanised FcγR models. However, single FcγR deficiencies may affect the expression of other FcγRs, such that interpretation may require caution [64].

1.4.3.1. Autoimmunity and inflammation

Fcgr2b-deficient mice have been crucial for establishing the key role of this receptor in the suppression of IgG-mediated immunity. FcγRIIB is essential for the maintenance of tolerance: KO mice develop increased autoantibody IgG titres with age [151], a phenotype shared with humanised mice containing the SLE-associated FcγRIIB-T232 polymorphism [179], and are susceptible to spontaneous autoimmune disease. This is most commonly associated with fatal glomerulonephritis [180]–[182]. Autoimmunity appears to be mediated by enhanced IL-17A production and could be abrogated through genetic deletion of the IL-17R adaptor protein CIKS, establishing a link between FcγR signalling and enhanced Th17 immunity *in vivo* [183].

As well as spontaneous autoimmune disease associated with FcγRIIB deficiency, passive transfer of IgG-containing immune serum or antigen immunisation leads to FcγR-mediated inflammation.

Fcgr2b-deficient mice are susceptible to collagen-induced (CIA) and antigen-methylated bovine serum albumin (BSA)-induced arthritis [184], [185]. Mice develop dysregulated B cell responses, elevated antigen-specific IgG titres, joint destruction, and leukocytic infiltration and activation [185]–[188]. Similarly, immunisation of *Fcgr2b*-deficient mice with bovine collagen type IV also resulted in pulmonary haemorrhage and glomerulonephritis in a murine model of Goodpasture's syndrome, a disorder characterised by IgG deposition on basement membranes [189]. FcγRIIB imparts protection from experimental autoimmune encephalomyelitis (EAE). EAE is a murine model of multiple sclerosis (MS) induced by immunisation with myelin oligodendrocyte glycoprotein in complete Freund's adjuvant [190]. In this model, *Fcgr2b*-deficient mice demonstrated by an increase in CNS demyelination [191].

These observations are often associated with the role of FcγRIIB on BCR activation. Strikingly, however, transfer of serum from arthritis patients was sufficient to induce arthritis in *Fcgr2b*-deficient mice, neatly demonstrating not only the pathogenic potential of IgG antibodies in RA, but also the B cell-independent roles for FcγR signalling in disease [192]. In the lung, passive transfer of ICs induces severe alveolitis in *Fcgr2b*-deficient mice, with increased haemorrhage, edema, neutrophil infiltration, and TNF production [193].

Fcgr2b-deficient mice were originally bred from back-crossing *Fcgr2b*-deficient mice on the 129-background with C57BL/6 mice, leading to transfer of additional susceptibility loci in the *Sle16* locus. *Fcgr2b*-deficient mice generated by gene targeting in B6-derived embryonic stem cells display similar enhanced susceptibility to autoimmunity, although with a less severe phenotype [194]. Therefore, FcγRIIB is essential for the maintenance of tolerance *in vivo*.

In contrast to *Fcgr2b*-deficient mice, mice deficient in activating FcγRs exhibited resistance to several models of arthritis [187], [195]–[198]. Furthermore, *Fcgr1*- and *Fcgr3*-deficient mice

show an impaired Arthus reaction, a vasculitis induced by injection of local antigen and systemic antigen-specific antibody [199]–[201]. Similarly, these mice are protected from rheumatoid factor-driven vasculitis, ascribed to impaired mast cell TNF production [202]. In contrast, *Fcgr4*-deficient mice are protected from a murine model of nephrotoxic nephritis, and are suggested to be the major conduit for IgG2b-mediated pathology in this model [197].

Transgenic mouse models allowing the cell-type specific modulation of FcγRIIB expression have allowed for the dissection of differential contributions of cell-mediated immune mechanisms towards IgG-mediated disease. FcγRIIB deletion at the germinal/post-germinal centre stage through Cg1Cre-mediated targeting demonstrate the important role of this receptor in inhibiting the development of spontaneous antibodies [203]. Furthermore, while B cell and DC-targeted FcγRIIB deletion enhanced susceptibility to CIA, only DC-specific FcγRIIB deficiency is relevant to K/BxN serum-induced arthritis, as expected [203]. Similarly, while B cell overexpression of FcγRIIB reduced IgG-mediated T cell responses and severity in CIA and SLE-like pathology [204], macrophage-specific FcγRIIB overexpression, in so-called macrophage-transgenic mice (M-TG), did not affect disease progression [205].

1.4.3.2. Infection

In addition to their ability to drive detrimental autoimmune disorders, FcγRs are essential mediators of immunity against a wide range of pathogens. *Fcgr2b*-deficient mice exhibit enhanced resistance to malaria and *S. pneumoniae*, with increased protective IgG responses, enhanced pathogen phagocytosis, and increased TNF and IL-6 production *in vivo* [163], [206]. Conversely, M-TG mice are more susceptible to *S. pneumoniae* infection and mortality [205]. *Fcgr1* and *Fcgr3*-deficient mice also show impaired responses to pathogen challenge, with increased bacterial burden and reduced cytokine production both *in vitro* and *in vivo* [207]–[209]. Therefore, the activity of both activating and inhibitory receptors is essential in mounting appropriate anti-pathogen responses.

One detrimental side effect of FcγR signalling in infection is illustrated in murine models of sepsis. While *Fcgr2b*-deficient mice are protected from lower levels of bacterial challenge, high bacterial burdens induce rapid mortality in *Fcgr2b*-deficient mice, attributed to an overwhelming inflammatory response [206].

1.4.3.3. Antibody-based therapy

FcγRs are essential for the *in vivo* efficacy of therapeutic monoclonal antibodies, for example in anti-tumour immunity and IVIG therapy, with many descriptions in the literature arising from the use of FcγR-transgenic mice.

In a murine model of syngeneic B16 melanoma, the efficacy of IgG2a monoclonal antibody therapy was increased in *Fcgr2b*-deficient mice, while several studies demonstrated reduced protection in the absence of individual activating FcγRs, although the specific contribution of each receptor varied between studies [124], [210]–[212]. Similarly, following anti-CD20 immunotherapy, murine lymphoma depletion was enhanced in the absence of FcγRIIB, and inhibited through combined deficiency of FcγRI, RIII, and RIV [213].

The protection of IVIG is intimately associated with FcγR function. Deletion of FcγRIIB reversed the therapeutic effect of IVIG in mice. This effect is thought to be driven by the upregulation of FcγRIIB expression in macrophages following IL-4 secretion by IL-33-activated basophils [196], [214]–[216]. While IVIG also downregulated FcγRIV expression, this was not sufficient to protect mice from nephrotoxic nephritis in the absence of FcγRIIB [217].

FcγRIII has also been demonstrated to mediate anti-inflammatory effects of IVIG. While FcγRIIB was required on host cells for IVIG protection, IVIG-primed donor DCs were dependent on FcγRIII for the suppression in a murine model of ITP [218]. Furthermore, FcγRIII-mediated IVIG signalling can downregulate IFNGR2 expression on host cells and inhibit NKT cells [219], [220].

Therefore, murine models demonstrate a role for FcγRs in the onset and progression of autoimmunity, and have allowed the mechanistic dissection of these effects otherwise impossible in human studies. Furthermore, studies have demonstrated that protective anti-microbial immunity and the response to therapeutic antibodies, from monoclonal antibodies to IVIG, are critically dependent on FcγR signalling *in vivo*.

1.4.4. Targeting IgG and FcγR signalling therapeutically

1.4.4.1. Targeting IgG and B cells

Strategies aimed at blocking or removing pathogenic IgG are commonly used in several inflammatory disorders. Plasmapheresis, the removal, treatment or exchange of blood plasma, is common in IgG-mediated disorders, such as ABMR [221]. IVIG is also used in ABMR to bind and remove pathogenic DSAs and prevent binding to target cells [222]. Enforced sialylation has been hypothesised as a mechanism by which to convert pathogenic IgG into an anti-inflammatory mediator similar to IVIG [137], and has been demonstrated to be effective in murine models of CIA [223]. However, the technical challenges for the translation of this method for the treatment of human disease are not clear.

As well as targeting IgG itself, rituximab, an anti-CD20 monoclonal antibody, is used to induce transient B cell depletion in autoimmunity, with B cell recovery typically within 6-9 months [222]. It is used effectively in the treatment of RA and off-label in SLE, as randomised controlled trials failed to show a benefit [224]. However, pan-B cell depletion can give rise to autoimmunity as

a result of regulatory B cell depletion. In contrast, BAFF inhibitors, such as belimumab, can deplete long-lived plasma cells and are used successfully in the treatment of SLE [225].

1.4.4.1. Targeting FcγRs

Strategies aimed at targeting FcγR-mediated inflammatory pathways have yielded success in the treatment of human autoimmune disease.

Small molecule SYK inhibitors were shown to be beneficial in RA patients [226]. However, given the widespread function and expression pattern of SYK, off-target side-effects are common and more targeted approaches are under development [227]. Modulation of FcγRIIB activity is central to many novel therapies. Enforced co-localisation of FcγRIIB with CD19 on B cells using an engineered anti-CD19 monoclonal antibody successfully suppressed humoral immunity in peripheral blood mononuclear cell (PBMC)-engrafted SCID mice [228]. Furthermore, small preliminary studies have demonstrated efficacy of soluble human FcγRIIB in the treatment of ITP and SLE [227].

Therefore, targeting IgG, B cells, and FcγRs have shown promise in the treatment of autoimmune diseases. However, sophisticated methods of manipulation are lacking at present.

1.5. The gastrointestinal tract and inflammatory bowel disease

1.5.1. Overview and host-microbe interactions

The GI tract comprises a series of organs whose primary functions are digestion, absorption, excretion, and protection. The stomach and small intestine (SI) contribute to physical and chemical digestion and absorption, while the LI is primarily involved in the desiccation and compaction of waste [229].

The GI tract is colonized by trillions of microorganisms that together form the microbiome, including at least 1000 species of bacteria, the major component of the commensal microflora. Bacterial colonisation increases progressively along the GI tract: while the stomach and proximal SI contains relatively low microbial densities (10^3-10^5 organisms per gram of luminal contents), the ileum (10^8 per gram) and colon (10^{10}-10^{12} per gram) are densely colonised, with greater species diversity. In particular, in the lower GI tract of healthy individuals, anaerobes dominate, including *Bacteroides*, bifidobacteria, fusobacteria, and peptostreptococci, while aerobes, such as enterobacteria, are present at lower densities [230].

During homeostatic conditions, a state of mutualism exists between the host and the commensal microbiota, whereby bacteria benefit from the energy-rich sources of food, and the host salvages essential compounds from indigestible nutrients, such as dietary polysaccharides [230]. Unsurprisingly, in such a scenario, the host immune system is skewed towards anti-inflammatory mechanisms that support ongoing symbiosis, with dynamic cross-talk between intestinal epithelial cells (IECs), microbes, and local immune cells preventing opportunistic invasion by commensal species [50], [231]. This includes the production of anti-microbial peptides and mucus by IECs, secretion and transport of IgA into the lumen, and an IL-10/TGFβ-dominated milieu that suppresses damaging inflammatory responses (Fig. 1.9). Conversely, immune education by intestinal microbes has been shown to be essential for adequate immune defence against mucosal-penetrating pathogens.

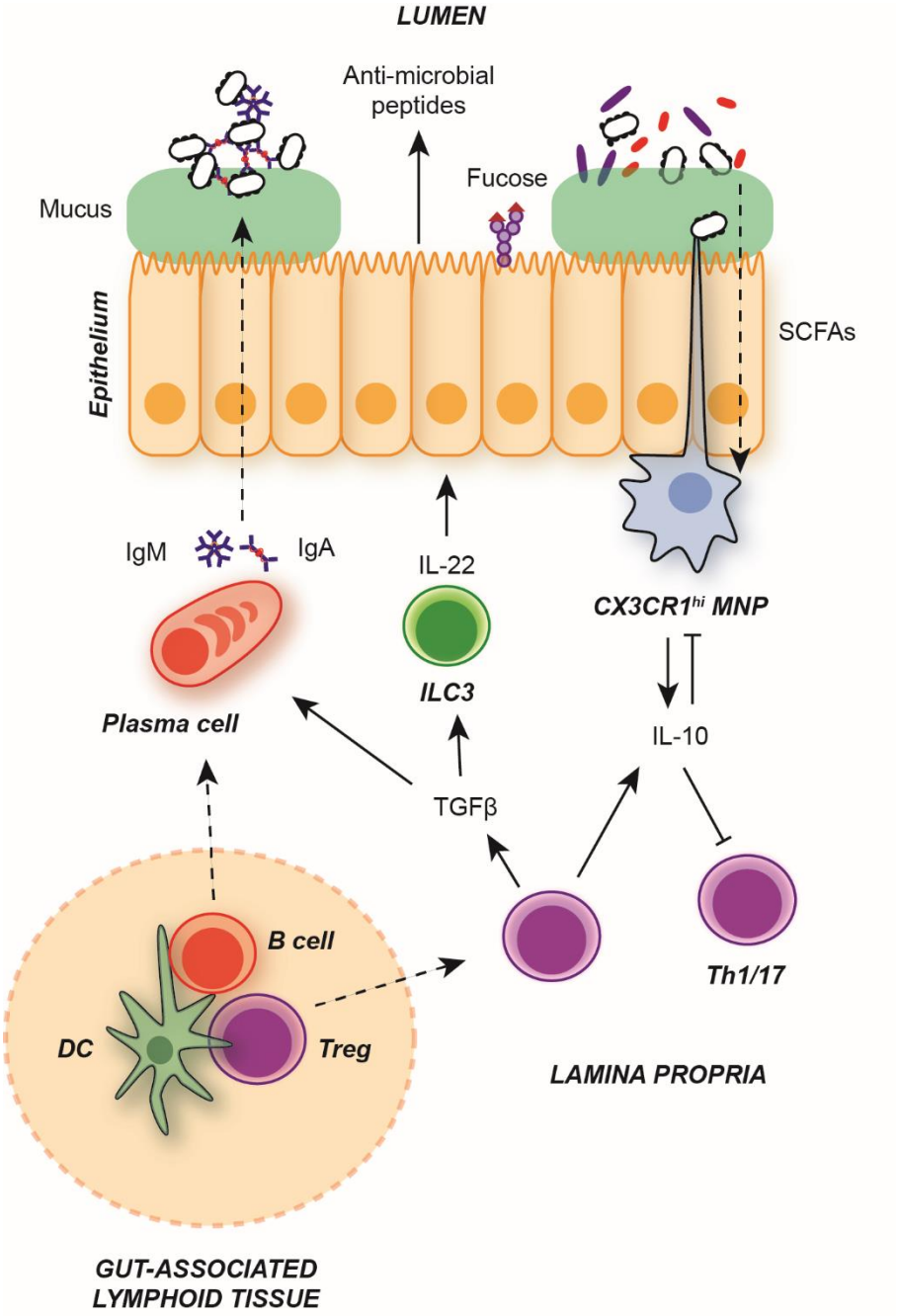

Figure 1.9. Homeostatic immune mechanisms that promote host-commensal mutualism in the GI tract. Several mechanisms contribute to the anti-inflammatory milieu in the GI tract. Tissue-resident plasma cells produce IgA to suppress bacterial immunogenicity and opportunistic invasion, ILC3-derived IL-22 promotes epithelial barrier function, and tissue-resident macrophages and DCs sample luminal contents, produce anti-inflammatory mediators, and induce GI-resident T cells. Bacteria influence immune cell phenotype through secretion of immunomodulators, such as short chain fatty acids (SCFAs).

1.5.2. Crohn's disease and ulcerative colitis

1.5.2.1. Overview

Inflammatory bowel disease is a chronic remittent or progressive inflammatory disorder of the GI tract that causes considerable morbidity and increased risk of colorectal cancer. There are two main subtypes, CD and ulcerative colitis (UC), that differ in their clinical and pathological presentations [232]. CD may affect any part of the GI tract and is associated with transmural inflammation affecting the entire mucosa and the formation of granulomas. In contrast, in UC, lesions are localized to the large bowel, resulting in continuous superficial mucosal inflammation and ulceration of the intestinal wall, with micro-abscess formation and neutrophil infiltration within the lamina propria (LP) common [233], [234].

While a strong genetic component exists, particularly in CD, the discordance among monozygotic twins (50-75 % concordance in CD; 10-20 % concordance in UC) and the increasing incidence of IBD in Western nations highlights the importance of environmental factors in disease development and progression [235], [236]. Strikingly, genome wide association (GWA) studies have demonstrated relatively modest risk associated with individual SNPs, suggesting that hundreds of polymorphisms may contribute to the onset of IBD [232].

1.5.2.1. Microbiota

Several circumstantial observations point towards the microbiota as being the target of the pathogenic immune response in IBD. As well as the association between several immune-sensing genes, such as *NOD2*, and disease susceptibility, IgG antibodies in IBD are commonly directed against components of the microbiota [237]–[242]. Furthermore, study of the microbiome has demonstrated that IBD itself is associated with decreased microbial diversity, the outgrowth of enterobacteria, and a reduction in anaerobes, such as Clostridia and Veillonellaceae [243]–[245].

How intestinal dysbiosis may initiate or propagate inflammation is the subject of intense research, not only within the intestine itself, but also for various systemic inflammatory disorders, and has benefited greatly from the use of sophisticated murine models, as shall be discussed.

1.5.2.2. Genetic associations

GWA studies have contributed significantly towards the understanding of the pathogenesis of IBD. Susceptibility is driven by a genetic predisposition to aberrant mucosal immune responses to commensals microbes, with unique and shared genetic traits between CD and UC (Fig. 1.10). Furthermore, inflammatory mechanisms in IBD are thought to be shared with

other extra-intestinal diseases, including ankylosing spondylitis and psoriasis, given the overlap of associations between these disorders [154], [246].

Prominent associations in CD include polymorphisms in *NOD2* and *ATG16L1* [234], [247]–[250]. NOD2 is an intracellular receptor for bacterial peptidoglycan [251]. Three uncommon SNPs in *NOD2*, located within the binding domain, result in reduced NOD2 function, with studies demonstrating that NOD2 signalling is required for the maintenance of tolerance and prevention of bacterial outgrowth within the GI tract [252], [253]. A SNP in the autophagy gene *ATG16L1* enhances its degradation and reduces autophagy [249]. This results in exacerbated endoplasmic reticulum (ER) stress within IECs, Paneth cell dysfunction, and exacerbated cytokine production [254]–[258]. These findings implicate an impaired capacity to sense bacteria and deal with ER stress as drivers of inflammation in CD.

UC, by contrast, is associated with defects in epithelial barrier integrity (*HNF4A* and *LAMB1*), IL-10 signalling, and the MHC locus [155], [259], [260]. Other than the MHC locus, one of the robust associations with UC is the FcγRIIA-H/R131 variant [155], [156], [261] (Fig. 1.10; bold italic). Homozygous R/R131 individuals are protected from the onset of UC (OR = 0.63; $P = 1.56 \times 10^{-12}$; [156]). This is in contrast to its association with SLE, in which R/R131 increases disease susceptibility and suggests that high-affinity variant H131 may contribute to disease progression through exacerbated inflammatory responses to IgG.

One of the key pathways implicated in both UC and CD is the IL-23/IL-17 cytokine axis [262]. An uncommon Arg381Gln substitution in the cytoplasmic domain of IL-23R confers significant protection against IBD, while alternatively spliced isoforms of *IL23R* transcripts are also linked to differential susceptibility. This has spawned an era of investigation into the role of IL-23 and other Th17-activating cytokines in GI inflammation and the development of novel therapeutics, as shall be discussed later.

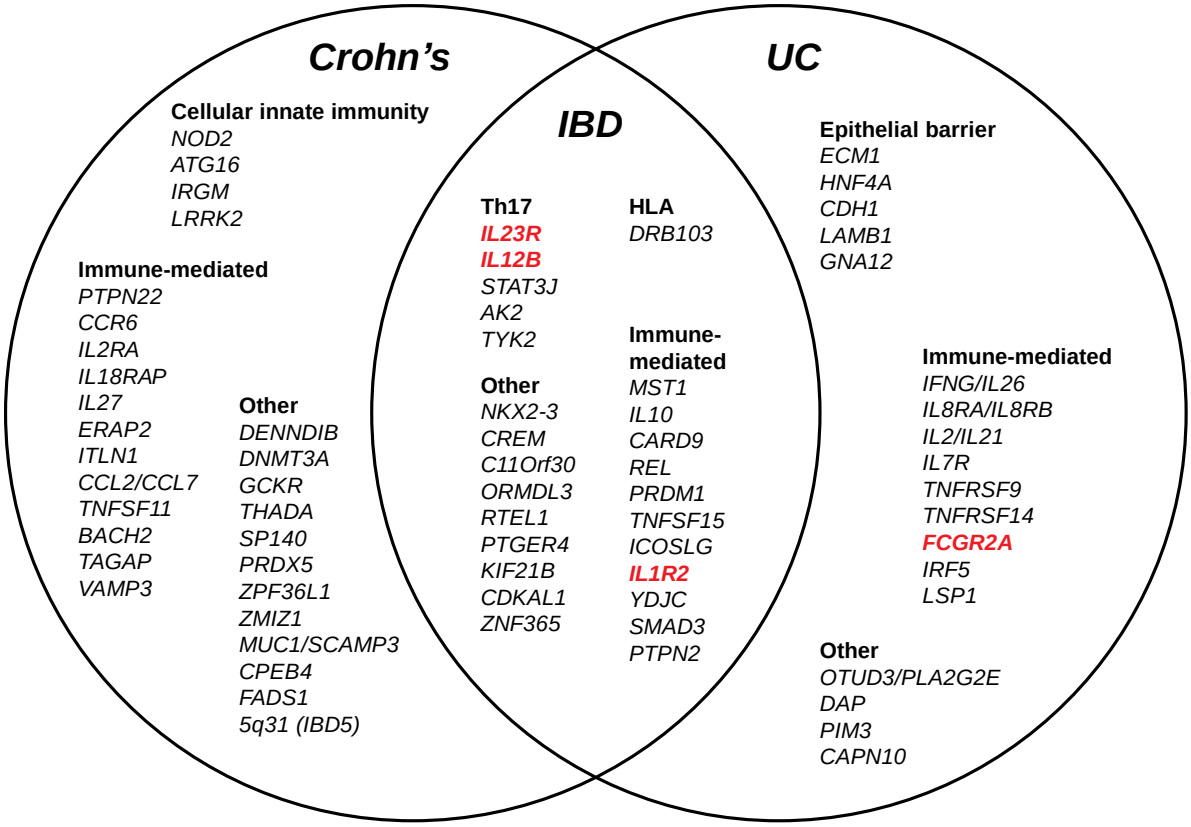

Figure 1.10. Genetic associations in IBD. IBD is prominently associated with defects in Th17 immunity. CD is specifically associated with defects in microbial sensing, such as *NOD2*, while UC is characterised by defects in barrier immunity and association with FcγR polymorphisms. Adapted from Lees et al. 2011 [263].

1.5.3. Murine models

Several murine models exist for the investigation of IBD, each with certain advantages and disadvantages. These include chemically-induced models of inflammation, including dextran sodium sulfate (DSS)- and 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis, infection models, such as *Citrobacter rodentium* and *Helicobacter hepaticus*, T cell-transfer models, and immune-activator models, such as anti-CD40 administration.

1.5.3.1. Chemically-induced colitis

Given its similarities in aetiology and pathology with human IBD, as well as its reproducibility and ease of use, DSS-induced colitis is perhaps the most prominent model of chemically-induced colitis. DSS is a colitogen with anticoagulant properties that erodes the epithelial monolayer of the colon when added to drinking water and can be administered for the study of both acute and chronic inflammation [264]. Clinical features include weight loss, shortening and thickening of the colon, blood in stools, and enlargement of spleen and mesenteric lymph nodes (MLNs) [265].

The duration of DSS administration dictates to a certain degree the clinical and immune profile associated with inflammation. Acute administration (5-7 days) is typically associated with epithelial destruction and ulceration, mucin and goblet cell depletion, and neutrophilic infiltration of the intestinal LP and crypts, features similar to human UC [266]. Furthermore, Th1 and Th17 cytokines are increased significantly [267]. In contrast, chronic DSS administration (2 or more cycles of 5-7 days) results in the infiltration of alternative pro-inflammatory immune cells, an increased prevalence of transmural inflammation and lymphoid follicle formation, characteristics of CD. While still prevalent, Th1 and Th17 cytokines are generally reduced relative to acute DSS, with an increase in IL-10 [267].

DSS administration for the study of intestinal inflammation is widespread, and has provided key translational insights into human disease, including the role of epithelial cells in barrier immunity [258], [268]–[273], the role of microbiota-derived metabolites [274], [275], intestinal humoral immunity [276], [277], and mechanistic insights into GWA study hits, such as those associated with the activation of Th17 immunity [82], [278]–[280].

TNBS-induced colitis is not as widely used, although has proved useful in the dissection of Th1-associated pathology. Used in combination with ethanol to disrupt the intestinal epithelial barrier, TNBS acts as a hapten by binding to proteins of high molecular weight, rendering them immunogenic [281]. TNBS induces transmural inflammation, reflective of CD-associated pathology, and high levels of IL-12 and IFN-γ production within the inflamed mucosa [267].

1.5.3.2. Infection models

Numerous infection models have contributed to our understanding of basic immunology within the GI tract and its translation to chronic inflammatory disease.

Citrobacter rodentium is a murine model of human attaching-effacing *Escherichia coli* infection [282]. Infection primarily targets the colon, with 10^9 organisms day 7-14 post-infection, and clearance of infection by day 21. Both B and T cells are essential, with IgG and IgM, not S-IgA, being required for sterilising immunity and the prevention of systemic bacterial spread [283], [284]. Furthermore, *C. rodentium* infection has gained popularity due to the requirement for Th17 immunity in its control, allowing for the study of IBD-associated mechanisms within the gut [285]–[291].

Helicobacter hepaticus has also proved useful in the investigation of intestinal Th17 immunity [292]–[297]. Although found in wild-type mice without any colitis, this enteropathogen can induce chronic proliferative typhlitis, colitis, and rectal prolapse in immunodeficient strains [298]. Indeed, *H. hepaticus* administration is usually carried out in immunodeficient *129SSvEv Rag2*$^{-/-}$ mice or in combination with anti-IL-10R administration. As such, its inability to successfully induce colitis in wild type (WT) mice restricts somewhat its applicability to bona

fide pathobionts in humans, but may more accurately reflect complications associated with dysbiosis and outgrowth of commensals during inflammation.

1.5.3.3. T cell transfer colitis

A widely used and cited experimental model of IBD is the adoptive transfer of naïve $CD4^+$ T cells ($CD4^+$ $CD45RB^{hi}$ T cells) into syngeneic immunodeficient mouse strains, such as SCID or *Rag1*$^{-/-}$ mice [299]. Following adoptive transfer, mice develop wasting disease and colonic inflammation after approximately 5-10 weeks. Histopathological analysis of inflamed colons demonstrates transmural inflammation, similar to CD, PMN and mononuclear leukocyte infiltration, crypt abscesses and epithelial erosions [300].

Transfer of mature $CD45RB^{lo}$ T cells does not induce colitis, an observation ascribed to the presence of Tregs. Indeed, not only was this model key in demonstrating the pathogenic role of T cells in colitis [301], but also the essential function of Tregs in suppression of intestinal inflammation [302]. As with other models, T cell-driven colitis is also dependent on the microbiota.

1.5.3.4. Anti-CD40 innate colitis

Another model making use of the absence of host adaptive immunity is the anti-CD40 innate model of colitis. In this model, *Rag1*$^{-/-}$ or SCID mice are injected with an agonistic anti-CD40 monoclonal antibody that induces an intestinal innate inflammatory response. CD40 is expressed by innate immune cells, such as DCs and monocytes, whereby it induces antigen presentation machinery, pro-inflammatory cytokine production, and proliferation [303]. Indeed, CD40-induced colitis is associated with elevated IL-23 production, making it useful for the study of the IL-23/IL-17A axis in relation to ILC-mediated pathology [304].

However, care must be taken in the observations made in immunocompromised mouse strains. The absence of local Tregs and IgA production likely has profound impacts on the microbiota and immune homeostasis within the gut, and probably contributes to the effectiveness of anti-CD40- or T cell-induced colitis.

1.5.4. Murine models and the microbiota

Studies in murine models of colitis have supported the notion that the microbiota has a predominant influence on intestinal disease development. GF mice are protected from several murine models of colitis, including DSS, while commensal-specific T cells can induce intestinal inflammation [237]–[242].

DSS and *C. rodentium*-induced colitis, as well as antibiotic treatment, causes dysbiosis that reduces the microbiota's ability to provide colonisation resistance. The subsequent outgrowth

of Enterobacteriaceae and enteropathogens can trigger inflammation, as well as supporting tumour development in mice [305]–[308]. Dietary fibre intake has also been shown to suppress outgrowth of mucolytic bacteria [309]. Finally, several examples exist demonstrating the ability of the microbiome to educate the intestinal immune system. Commensal-derived SCFAs induce colonic Tregs and promote IEC function, bacterially-induced Th17 cells regulate EAE susceptibility, while faecal transplantation alters murine susceptibility to colitis and obesity [274], [277], [310]–[316].

Therefore, while host-microbe interactions are a key facet of intestinal pathology, it is clear the majority of commensal microbes are likely to be host protective, and the distribution and composition of the microbiome skewed in disease [317].

1.6. Immunopathology in inflammatory bowel disease

1.6.1. Overview

The host mechanisms that drive intestinal inflammation are under intense investigation given the clear involvement of the immune system in disease. In addition to the association with IL-23/Th17 axis, both CD and UC are characterised by the altered production of inflammatory cytokines [318]. For example, CD is associated with the production of IFNγ and Th1 cytokines, driven by IL-12 signalling, while in UC, LP cells produce elevated levels of IL-5 and IL-13, classically Th2-associated cytokines [319], [320]. However, these rigid classifications of IBD-associated inflammatory networks are oversimplifications of a broader network of immune dysfunction. Furthermore, the functional relevance of certain observations has proved enigmatic with regards to translation to the clinic. Here, we discuss some of the major pathways implicated in IBD pathology, how murine models have informed our understanding of disease, and attempts to target them therapeutically.

1.6.2. Antigen-presenting cell-derived cytokines

Dysregulated cytokine production by myeloid cells, in particular APCs such as macrophages and DCs, is observed in patients with IBD. Indeed, APC-derived cytokines currently represent the most successful therapeutic targets in this disease. Here, our current understanding of the mechanisms of action of these cytokines and the implications for murine and human disease pathology are discussed.

1.6.2.1. Tumour necrosis factor and TNF ligand-related molecule 1A

TNF is a pleiotropic heterotrimeric cytokine. It is first produced as a transmembrane protein before being cleaved by the metalloproteinase TNF-converting enzyme. TNF exerts its functions through two receptors: TNF receptor 1 (TNFR1) is ubiquitously expressed and drives

pro-inflammatory responses, while TNF receptor 2 (TNFR2) expression is more restricted and associated with homeostasis [321].

TNF induces various inflammatory mechanisms, such as augmentation of angiogenesis, cellular apoptosis and necroptosis, with the concomitant release of intracellular contents [322], MMP production, and immune cell activation [318], [323]. However, the role of TNF is complicated given its involvement in homeostasis. For example, TNF can desensitise macrophages [324], induce IL-10 production, and promote inflammatory cell senescence and death [325]. Furthermore, TNF is required for appropriate formation of B cell follicles and GCs [326]. Therefore, therapeutic targeting of TNFR1 may help to refine anti-TNF therapy to avoid undesirable complications [321].

In IBD, aberrant TNF production is observed in the colonic mucosa. CD-like models of colitis support a role for TNF in driving inflammation: overexpression of TNF in mice results in spontaneous transmural inflammation, dependent on TNFR1, IL-12p40 and IFN-γ production by T cells [327], [328]. Similar observations are seen in the *T-bet*$^{-/-}$ x *Rag*$^{-/-}$ ulcerative colitis (TRUC) model – communicable colitis in TRUC mice is associated with exacerbated TNF production by DCs [329], [330]. Furthermore, macrophage and IEC-derived TNF drives intestinal inflammation in the CD45RB^{hi} transfer model of colitis [331]. In relation to its regulating cell death, two studies demonstrated that TNF-driven IEC apoptosis and necroptosis is a key driver of colonic inflammation [332], [333]. Recently, TNF was shown to synergise with OSM, a cytokine recently identified as a driver of infliximab-resistant IBD, for the induction of pro-inflammatory chemokines and cytokines [334].

In contrast, in DSS-induced colitis, TNF-deficient mice are more susceptible to disease. Given the similarities of DSS-induced colitis to UC, this may represent a differential role of TNF in different models of colitis [335]. Alternatively, this may reflect a requirement for homeostatic TNF levels within the gut, with exacerbated TNF production remaining pathological.

Regardless, anti-TNF monoclonal antibodies are currently the most efficacious treatment for IBD, with infliximab administration a cornerstone of IBD therapy [232]. Infliximab is an IgG1 chimeric antibody that binds to both soluble and membrane TNF. In contrast, etanercept, a soluble TNFR fused to IgG1 Fc, has little therapeutic effect in IBD, activity ascribed to its inability to block membrane TNF and kill LP-resident T cells [336]. Studies have demonstrated that the *in vivo* efficacy of infliximab is dependent on FcγR binding [337], and the ability of TNF-infliximab interactions to from anti-inflammatory ICs that dampen disease locally, given the heterotrimeric nature of TNF (van den Brink; unpublished communication). This may explain which etanercept, which cannot form ICs, is less effective.

TNF ligand-related molecule 1A (TL1A) is also increased in CD and associated with DSS-induced colitis [338]–[340]. Functionally, TL1A is derived from DCs and macrophages and

promotes IFN-γ production by T and NK cells, while recent studies have also implicated TL1A in ILC activation in the gut [291]. This once more reinforces the notion of Th1 characteristics in CD. However, targeting of TL1A in human disease has yet to be carried out.

Therefore, elevated TNF responses appear to contribute to intestinal disease development, particularly in murine models resembling CD-like pathology, in which Th1 responses are exacerbated. Furthermore, targeting this pathway is currently the gold standard approach to treating IBD, although the specific targeting of TNF may prove to be somewhat circumstantial.

1.6.2.2. IL-1β

IL-1β is a prototypical inflammatory cytokine with wide-ranging effects on virtually all cells and organs [341]. Unsurprisingly, therefore, it is a major pathogenic mediator of inflammation.

Unlike the alarmin IL-1α, which is constitutively produced by many non-haematopoietic cells, IL-1β is produced by haemtopoietic cells is response to PAMP/DAMP, activated complement, or cytokine stimulation. It requires cleavage from an inactive pro-form by caspase 1 for extracellular release. Pro-caspase-1 itself also requires activation, which occurs via the assembly of inflammasome complexes within cells. A key component of inflammasomes is nucleotide-binding oligomerisation domain, leucine-rich repeat and pyrin domain containing 3 (NLRP3) [341], [342]. However, non-canonical inflammasome-mediated activation via caspase 11 is also possible, as well as extracellular activation of pro-IL-1β by neutrophil enzymes. Metabolic changes in activated immune cells can also support IL-1β production. For example, accumulation of succinate in macrophages stabilises hypoxia-inducible factor 1 alpha (HIF-1α), which induces pro-IL-1β [343].

IL-1β plays pleiotropic roles in inflammation. It acts as the classic endogenous pyrogen to induce fever and promotes IL-6 expression for induction of the acute phase response in the liver [344]. IL-1β also influences neutrophil inflammatory responses, driving immune cell activation, the upregulation of adhesion molecules on endothelial cells, and prolonging the lifespan of leukocytes within inflamed tissues [341], [345]. Finally, IL-1β has important roles in type 17 immunity, as shall be discussed in the next section.

Elucidation of the role of IL-1β in IBD has lagged behind IL-23. However, promising results demonstrate this may represent a novel target for therapeutic intervention. Enhanced IL-1β production is associated with IBD pathogenesis, with IL-1β expression increased in the inflamed colonic mucosa relative to levels of IL-1 receptor antagonist (IL-1Ra) [346]. Furthermore, IL-1β levels are enriched in colonic biopsies from patients with infliximab-refractory IBD [334]. Encouragingly, targeting IL-1β signalling has proved fruitful in several murine models of colitis. NLRP3 inflammasome-dependent IL-1β production is induced by distinct commensal microbes and its blockade has proved beneficial in DSS-induced colitis

and *H. hepaticus* infection [279], [296]. Furthermore, caspase-1-deficient mice are protected from DSS-induced inflammation [347].

Therefore, given its pleiotropic roles in inflammation, and recent demonstrations of dysregulated IL-1β responses in human and murine colitis, IL-1β targeting likely represents one of the major novel avenues to be exploited for IBD therapeutic intervention. Indeed, trials of IL-1Ra in UC are currently underway (Arthur Kaser, unpublished communication).

1.6.2.3. IL-23

IL-23 is a heterodimeric cytokine composed of the IL-12p40 subunit, shared with IL-12, and IL-23p19 (otherwise known as IL-23A). Since its discovery in the early 2000s, particular attention has focused on its role in the activation of immune cells that dually express IL-23R and RORγt, including Th17 cells, group 3 ILCs (ILC3s), and γδ T cells. In response to IL-23, these cells potently produce signature type 17 cytokines, including IL-17A, IL-22, and granulocyte macrophage-colony stimulating factor (GM-CSF), that contribute to local tissue inflammation [348].

In many circumstances, IL-1β and IL-23 act in concert for the activation of type 17 immunity. Our current understanding of Th17 biology suggests that while IL-23 can promote Th17 differentiation and survival from naïve $CD4^+$ T cells along with TGFβ and IL-6 [349], IL-1β promotes the expansion and maintenance of Th17 cells in inflamed tissues following IL-6-induced IL-1R1 upregulation [350]. Mechanistically, signalling via IL-1R1 induces expression of transcription factor interferon-regulatory factor 4 (IRF4) via phosphorylation of mammalian target of rapamycin (MTOR), which in turn reinforces expression of RORγt [351]. Strikingly, O'Shea and colleagues demonstrated that combined stimulation of naïve T cells with IL-23, IL-1β, and IL-6, in the absence of TGFβ, was sufficient to induce a pathogenic subset of Th17 cells, characterised by expression of RORγt, T-bet, and IL-33 [352]. Therefore, cooperation between IL-23 and IL-1β likely exacerbates type 17 immune responses.

As well as Th17 cells, IL-1β and IL-23 support innate cytokine production by γδ T cells and ILC3s, likely bridging the temporal gap prior to the onset of adaptive immune responses [353]–[358].

Several studies, in both WT and *Rag2*-deficient mice, have supported a pathogenic role for IL-23 in intestinal inflammation. IL-23 was shown to drive pathology in two models of *H. hepaticus*-induced T cell-dependent colitis independently of IL-12, whereby IL-17A and IFNγ synergized to promote tissue destruction. Monoclonal anti-IL-23p19 IgG administration was sufficient to control colitis in this case. Furthermore, several studies have shown that IL-23 can drive innate type 17 cytokine production and IFNγ in both adult and neonatal intestines that are sufficient to promote microbiota-dependent colitis [292], [294], [295], [359]–[362]. In

contrast, IL-23 exerts a protective role in TNBS-induced colitis by blocking IL-12 secretion [363].

Excitingly, anti-IL-12p40 and anti-IL-23p19 monoclonal antibody therapy significantly increased clinical remission rates in moderate-to-severe CD patients that were resistance to infliximab [364], [365], and nicely illustrates how murine models have helped to inform human pathology.

Therefore, the concerted action of IL-23 and IL-1β supports intestinal pathology through the activation of type 17 cytokine responses and IFNγ production, with clinical intervention of these pathways proving to be promising in both human disease and murine colitis.

1.6.3. T and innate lymphoid cell-derived cytokines

Type 17 responses are of major interest in the field of IBD research, given the abundance of studies demonstrating the therapeutic benefit of IL-23 and IL-1β blockade in both mice and humans. However, the specific roles of these cytokines, particularly IL-17A, IL-22, and GM-CSF, remain enigmatic. Indeed, little or no therapeutic benefit has been demonstrated from targeting these cytokines individually.

1.6.3.1. IL-17A and IFNγ

Since the discovery of Th17 cells and their association with several inflammatory disorders, studies probing the biology of IL-17A have been widespread. IL-17A seems to be of particular significance at epithelial barrier sites, where Th17 cells, γδ T cells, and ILC3s reside in abundance. Here, IL-17R signalling is thought to drive tissue destruction through its ability to promote cytokine production and cellular recruitment via the activation of local non-haematopoietic cells [366].

Mechanistically, IL-17A can induce cytokine production from both haematopoietic and non-haematopoietic cells, including IL-1β, IL-6, GM-CSF, PGE2 and TNF [366]–[369]. IL-17 also has a critical role in neutrophil recruitment to tissues via the induction of chemokines, including CXCL1, CXCL2 and CXCL8, while GM-CSF and MMP induction can sustain neutrophil survival and activation within tissues [370]–[372]. At mucosal sites, IL-17 can also induce expression of anti-microbial peptides critical for barrier immunity [373], [374].

In many instances, IL-17A also acts cooperatively with other cytokines, particularly TNF. IL-17A can induce expression of TNFR1 and acts in synergy to drive production of anti-microbial peptides, chemokines, IL-1β and MMPs [375], [376]. Furthermore, both cytokines induce endothelial cell expression of selectins, CXCL1 and CXCL2 for sustained neutrophil recruitment to inflamed sites.

The involvement of Th17 cells in inflammation appears to be widespread, from anti-microbial immunity [367] to autoimmune and inflammatory disorders, such as psoriasis [377], RA [378], MS [379], asthma [380], SLE [381], and IBD [262]. Furthermore, γδ T cell-derived IL-17A has been shown to promote neutrophil infiltration following *E. coli* infection [382], and to augment the severity of EAE [354]. γδ T cell-derived IL-17A can also sustain IL-23-driven dermal inflammation in Imiquimod models of psoriasis [383].

Within the GI tract, IL-17A has been shown to be necessary for defence against intestinal pathogens, such as *C. rodentium* [384]. However, the role of IL-17A in driving colitis remains enigmatic: IL-17A neutralisation exacerbates DSS-induced colitis [385] and monoclonal anti-IL-17A antibodies enhance IBD severity [386]. This has been hypothesised to be due to the roles of IL-17A in the preservation of the intestinal epithelial barrier. Lee et al. found that γδ-T cell production of IL-17A was independent of IL-23 and associated with a reinforced epithelial barrier [387], while Maxwell et al. demonstrated that IL-17RA blockade, although reducing neutrophil recruitment during colitis, resulted in an exacerbation of colitis and reduced epithelial barrier function [388]. Finally, Song et al. demonstrated a role for IL-17A in epithelial wound healing following DSS-induced colitis [389]. Therefore, while IL-17A is clearly associated with colitis and is involved in neutrophil recruitment to the inflamed GI tract, these studies argue for IL-17A-independent roles for IL-23 in the exacerbation of disease.

IL-23 can induce IFNγ secretion by inflamed colonic LP cells, and argues against a strict Th17 role for IL-23 [390]. IFNγ contributes to inflammation through the activation of tissue-resident macrophages, apoptosis of IECs, and augmentation of antigen processing [318]. Elevated IFNγ expression is historically characteristic of LP-resident $CD4^+$ T cells [335]. In CD patients, IFNγ production vastly outnumbers IL-17A production, and is strongly linked to disease activity [391]. Initial studies in the $CD4^+$ T cell-transfer model of colitis demonstrated that blockade of IFNγ or T-bet deficiency (a transcription factor required for IFNγ production) was sufficient to suppress inflammation [301], [392]. Similarly, IFNγ-deficient mice are protected from DSS-induced colitis [393]. Given the observation of T-bet-expressing Th17 cells, it may be possible that these pathogenic T cells contribute to inflammation through dual IL-17/IFNγ production.

Strikingly, a study by Feng et al. demonstrated that transfer of flagellin-specific CBir Th17 cells into T cell-deficient mice resulted in IL-17-dependent innate IL-12 and IL-23 production and the emergence of pathogenic IFNγ-producing Th17 cells [394]. In this model, blockade of IL-17A was effective in the suppression of colitis. Therefore, it is possible that IL-23-dependent mixed Th1/Th17 responses cooperate in the induction and progression of colitis. However, common to IL-17A, the IFNγ-specific monoclonal antibody fontolizumab has not proved effective in CD therapy [318], [395].

Therefore, despite associations between upstream innate cytokines and the activation of Th1 and Th17 immunity in the GI tract, as well as the clear roles played by T cells in driving colitis, targeting single Th-associated cytokines has not proved effective in the treatment of IBD.

1.6.3.2. IL-22

IL-22 is a recently described cytokine of the IL-10 family that is produced by a variety of cells, including DCs, NKT cells, γδ T cells, Th17 cells, and the newly identified ILC3s [396].

IL-22 receptor expression is largely confined to non-haematopoietic cells, particularly epithelial and stromal cells, where it signals via STAT3 to induce a programme of cellular proliferation, tissue regeneration, and reinforced barrier function [397]. Therefore, unsurprisingly, IL-22 plays a key role at mucosal interfaces, with a wealth of growing data as to its role in the GI tract.

In vivo, IL-22 production by T cells and ILC3s is heavily dependent on IL-23, with increased numbers of IL-22-producing ILC3s observed in patients with IBD [398]. However, despite its link to IL-23, the roles of IL-22 in driving colitis are unclear. In chronic $CD45RB^{hi}$ $CD4^{+}$ T cell transfer and DSS-induced models of colitis, IL-22 was protective [361], [399]–[402]. However, blockade of IL-22 in acute CD40-induced colitis was beneficial, although the mechanism by which this was achieved was unclear [361]. Indeed, Powrie and colleagues initially ascribed the pathogenic role of $CD90.2^{hi}$ $SCA\text{-}1^{+}$ ILCs in this model of colitis to their production of IL-17A and IFN-γ, rather than IL-22 [292].

While its role in driving colitis is disputed, IL-22 may be involved in IBD-associated colorectal cancers through its ability to stimulate epithelial cell turnover. Indeed, murine models have demonstrated sustained *H helicobacter* + azoxymethane (AOM)-driven colorectal cancer through an IL-22-dependent mechanism [403]. IL-22 activity *in vivo* can be antagonised by IL-22 binding protein (IL-22BP), highly expressed by intestinal DCs and dynamically regulated during immune responses. In support of a tumorigenic role for IL-22, IL-18-dependent downregulation of IL-22BP is associated with enhanced tumorigenesis in the DSS/AOM model of colorectal cancer [404].

In summary, IL-22 appears to exert a homeostatic function in the repair and maintenance of the intestinal epithelium, with little direct association with IBD pathology. However, dysregulated IL-22 activity may contribute to IBD-associated colorectal cancer. The regulation and function of IL-22 will be discussed in more detail in the section on innate lymphoid cells.

1.6.3.3. Granulocyte-macrophage colony-stimulating factor

GM-CSF is a member of the CSF superfamily that signals through a heterodimeric receptor composed of the GM-CSF-specific α chain (CSF2RA) and the signal transducing β subunit

(CSF2RB). GM-CSF is produced in response to danger signals by many cell types, including DCs, T cells, B cells, ILCs, and non-haematopoietic cells [405], [406]. As well as its well established roles in the myelopoiesis, recent reports have demonstrated key inflammatory roles for GM-CSF in mediating tissue destruction, with implications for IL-23- and IL-1β-driven inflammation [407].

GM-CSF has been identified as an M1-inducing stimulus that can induce inflammatory reprogramming of macrophages that may drive local inflammation and immune defence [72], [408], [409]. GM-CSF can promote LPS-induced inflammatory cytokine production by BMDMs *in vitro,* including IL-1β, a feature associated with enhanced glycolytic flux and expression of the GTPase Rab39a [410], [411]. Indeed, GM-CSF has been shown to drive EAE via the licensing of pathogenic IL-1β production by $CCR2^+$ monocytes [412]. Furthermore, GM-CSF can sustain neutrophil survival and activation in the periphery, as previously mentioned.

Higher levels of GM-CSF are identified in mucosal lesions in CD and UC compared to healthy controls. Furthermore, colonic GM-CSF expression is increased following DSS exposure [413].

In the murine *H. hepaticus* colitis model of IL-23-driven colitis, GM-CSF can drive dysregulated haematopoiesis and local granulocyte activation to promote colitis [414], [415]. Furthermore, GM-CSF-deficient mice are more susceptible to *C. rodentium* infection. Conversely, however, GM-CSF depletion exacerbates DSS-induced colitis, ascribed to defects in DC and macrophage survival and activation [413], [416], [417].

Therefore, GM-CSF clearly has roles in the maintenance and activation of myeloid cells within the GI tract, although the relative contributions to homeostatic and inflammatory programmes likely differ between models. It is noteworthy that anti-GM-CSF antibodies are identified in a subset of patients with IBD (particularly CD), and associated with enhanced disease severity, intestinal epithelium permeability and reduced neutrophil responsiveness [418]–[420]. In this setting, it is tempting to speculate this may contribute to colitis as a result of dysbiosis and opportunistic invasion by commensal microbes, in a manner similar to NOD2 polymorphisms.

1.6.4. IL-10

IL-10 is the prototypical anti-inflammatory cytokine. Most classically associated with $Foxp3^+$ Tregs, IL-10 functions to suppress lymphocyte and myeloid immunity through a variety of mechanisms, such as the inhibition of inflammatory cytokine production [421].

Within the GI tract, IL-10 is also produced by macrophages, regulatory B cells (Bregs), and epithelial cells [5], [422]–[424]. Here, the critical role of this cytokine is acutely illustrated by associations between *IL10R* SNPs and UC, as well as the widespread use of the *H. hepaticus*

infection model: the presence or absence of IL-10R signalling dictates whether an otherwise innocuous bacterium induces colitis [259], [260], [298].

A recent study by Medzhitov and colleagues demonstrated that IL-10R signalling in BMDMs was essential for the inhibition of glycolysis and the clearance of damaged mitochondria by mitophagy. In the absence of IL-10R signalling, macrophages accumulated damaged mitochondria in a murine model of colitis and in patients with IBD, resulting in exacerbated NLRP3-dependent IL-1β production [425]. A complementary study also demonstrated that IL-10Rα signalling in intestinal macrophages was required for the suppression of intrinsic IL-1β production via transcriptional suppression of the *Il10* locus and caspase-1-dependent pro-IL-1β processing [426].

Mauri and colleagues demonstrated that microbiota-dependent IL-6 and IL-1β production was sufficient to induce IL-10-producing Bregs within MLNs that contain systemic inflammation [423], while adoptive transfer of these cells is sufficient to suppress intestinal inflammation [422].

Therefore, through its roles in the suppression of intestinal macrophage function, IL-10 is critical for the maintenance of a tolerogenic milieu within the GI tract.

1.6.5. IgG and FcγRs in IBD

Given the prevalent inflammatory characteristics of IgG, it is unsurprising that *de novo* IgG generation is associated with chronic intestinal inflammation in IBD patients [240], [427]. This IgG response is directed against components of the commensal microbiome, with a prominent example of an IBD-associated antigen being flagellin [241], [428], [429]. It has been demonstrated that $CXCR4^+$ IgG plasmablasts are enriched in the inflamed colonic LP of patients with IBD [430], while additional inflammatory mechanisms have been ascribed to LP-infiltrating plasma cells, such as granzyme B production [431].

UC patients are also characterised by the development of auto-antibodies, resulting in its classification as a partially autoimmune disorder. Circulating antibodies against colon epithelial goblet cells are common [432], while perinuclear anti-neutrophil cytoplasmic antibodies (pANCA) are observed in two thirds of UC patients [433]–[435].

However, despite the robust association between FcγRIIA SNPs and IBD, a systematic characterisation of IBD-associated IgG is severely lacking. Furthermore, the pathological implications of this local antibody production remain largely unknown.

Recently, aberrant IgG glycosylation was demonstrated in IBD patients, with agalactosyl IgG associated with both UC and CD [436]. Indeed, 5 genes known to regulate IgG glycosylation show robust association with IBD (*IKZF1*, *LAMB1*, *MGAT3*, *IL6ST*, and *BACH2*) [127],

including genes encoding galactosyltransferases [126]. Given the association between pathological IL-23R signalling and IBD, and the observation that IL-23-derived Th17 immunity promotes IgG class-switching and inflammatory glycosyl patterns, these pathways may reinforce one another for the augmentation of pathology in the GI tract.

Drugs targeted at reducing IgG-mediated inflammation have received little attention in the treatment of IBD. No randomised control trials exist for IVIG but a meta-analysis identified a handful of case reports which indicated that IVIG can induce a rapid improvement in steroid resistant CD [437], [438], and there are reports of utility in UC [439].

A single randomised controlled trial exists for the anti-CD20 antibody rituximab in UC [440]. Of 16 patients who failed to respond to standard therapies, half demonstrated a response at 4 weeks compared to 2 of 8 placebo-treated patients. This response was not maintained to 12 weeks in a further half of patients, however. The small size of this study, however, is in stark contrast to the cohort size required to demonstrate efficacy of other IBD therapeutics [365], and was substantially underpowered. Furthermore, given the likely depletion of regulatory B cells with rituximab, pan-B cell depletion can result in exacerbation of allo- and auto-immunity, and my not be the optimal therapeutic strategy. Therefore, the question of whether B cell manipulation may be of benefit in UC has not been adequately addressed.

These observations challenge the dogma that IgA is the predominant method of humoral protection at mucosal surfaces. Additionally, FcγR signalling at mucosal sites in response to local IgG could drive disease pathology in IBD. Given its induction *in vitro* following FcγR ligation, this may proceed through the activation of type 17 immune responses within the inflamed mucosa. This shall be discussed in more detail below.

1.7. Intestinal B cells

Intestinal plasma cells are an essential component of mucosal immunity, with the majority of plasma cells producing IgA under homeostatic conditions [42]. Secretory IgM-producing plasma cells are also present in the human gut [441], and can survive for decades within the GI tract itself, as well as in the bone marrow [442], [443]. During inflammatory disorders, such as IBD, IgG-producing plasma cells are markedly increased, demonstrating a skewing of the humoral response from an anti- to a pro-inflammatory phenotype [430]. However, the mechanistic underpinning and consequences of this switch are not well understood.

Here we shall discuss the mechanisms of Ig within the GI tract, as well as their generation during homeostasis and inflammation.

1.7.1. IgA in homeostasis

S-IgA participates in the maintenance of mutualism in the GI tract through a variety of mechanisms collectively known as immune exclusion.

S-IgA can neutralise toxins and pathogens without causing inflammation because of its inability to fix complement [444]. In addition, S-IgA anchors microbes to the mucus, preventing invasion of the underlying epithelium. S-IgA can suppress inflammatory responses to bacterial products: it downregulates expression of inflammatory epitopes [445] and can neutralise intracellular LPS within IECs [446]. Via its ability to agglutinate bacteria through carbohydrates in the Fc domain, S-IgA induces the formation of a biofilm that inhibits the growth of pathogens [447]. Recently, high affinity IgA was shown to mediate enchainment of bacterial daughter cells as they divide, limiting colonisation, bacterial evolution, and promoting elimination from the body [448]. This function of high-affinity TD IgA may allow for the retention of beneficial commensals, which do not elicit such a potent response under homeostatic conditions. Finally, natural or low-affinity IgA can deliver antigens to M cells for transport into Peyer's patches (PP) for the induction of adaptive immune responses [449].

The importance of IgA is demonstrated by species of commensal microbes that mediate IgA degradation, which promote GI inflammation [276].

1.7.2. CSR in the GI tract

Gut-draining MLNs, gut-associated lymphoid tissues (GALT), including PP and isolated lymphoid follicles (ILFs), and the intestinal LP have been proposed to be sites for IgA generation, with the mode of IgA generation differing across tissues [450]. Local IgA production within the LP and ILFs is thought to be largely TI, given the absence of abundant T cell zones, whereas MLNs, the caecal patch, and PPs can support both TD and TI IgA induction [42], [451], [452].

Generally, IgA generation in T cell-deficient mice is less efficient and less effective than in WT mice [45]. Furthermore, while T-B cell interactions do not appear to be required for CSR, the vast majority of LP-resident plasma cells are heavily mutated, suggesting a germinal centre origin, T cell help, and SHM [453].

1.7.2.1. IgA generation in GALT

PPs are the major source of intestinal IgA^+ B cell induction (Fig. 1.11) [453]. These structures are composed of several B cell GCs on a network of FDCs, with interfollicular regions containing T cells and DCs. Tfh cells are essential for IgA class-switching, and can arise from the plasticity of other $CD4^+$ T cell subsets within PPs, including Tregs and Th17 cells [454]–[457]. $CXCR5^+$ $PD\text{-}1^{hi}$ $FoxP3^+$ T follicular regulatory cells are required to limit Tfh numbers

and dampen GC B cell responses in order to maintain commensal microbiome fitness [456]–[458].

While CD40-deficient mice exhibit normal levels of IgA-producing cells, in the absence of GCs, these cells have not undergone SHM, as in WT mice [240], [459]. Furthermore, *Aicda*-deficient mice and AID^{G23S} knock-in mice (with a specific defect in SHM) exhibit intestinal dysbiosis, B cell hyperplasia, bacterial translocation, and susceptibility to enteropathogenic challenge [460], [461]. Therefore, while T-B interactions are not required for low-affinity TI IgA responses, they are required to mount specific IgA responses to microbes and orally administered T-dependent antigens. Non-cognate T-B interactions have been shown to be sufficient for GC formation and SHM in PPs. B cells expressing non-antigen-specific surrogate Ig receptors can induce GC formation in PPs, dependent on non-cognate T cells and the microbiota [462]. Therefore, under certain circumstances, BCR-independent B cell activation can support TD IgA production in PPs.

Other than T cells, other PP-resident haematopoietic and non-haematopoietic cells influence IgA class-switching and subsequent LP-homing, including DCs and FDCs. Gut-imprinting via the upregulation of CCR9 and α4β7 is mediated by retinoic acid (RtA)-producing GALT DCs [463], [464]. A recent study by Reboldi et al. demonstrated that CCR6-dependent B cell migration to RtA-producing DCs within the PP sub-epithelial dome was required for IgA class-switching. In the absence of CCR6, antibody production was skewed towards IgG1 [8]. PPs DCs are also a source of NO, which has also been demonstrated to promote both TD and TI IgA CSR in GALTs via regulation of B cell TGF-β receptor expression, and the production of APRIL and BAFF, respectively [52]. Indeed, TGFβ can support CSR to IgA *in vitro*, and TGFβ receptor-deficient mice do not generate IgA-producing plasma cells following immunisation [465].

FDCs have also been implicated in IgA CSR. As well as presenting antigen to B cells within follicles, FDCs produce CXCL13, BAFF, and molecules that facilitate the secretion and activation of TGF-β1 in response to microbial stimuli and RtA [53].

1.7.2.1. IgA generation and maintenance in the lamina propria

Whether CSR occurs in the intestinal LP is disputed. While certain CSR-associated factors are expressed within the LP in humans and mice, convincing evidence of AID expression by LP-resident plasma cells is lacking. Instead, the intestinal mucosa may provide local niches that support plasma cell survival (Fig. 1.12) [42]

Intestinal DCs and IECs can contribute to plasma cell survival through production of IL-6, CXCL12, BAFF and APRIL [466]–[468]. Eosinophils are abundant in the GI tract, although their local functions are not well understood. *In vitro,* eosinophils can support IgA class-

switching, while eosinophil depletion *in vivo* resulted in defects in TGFβ-mediated events, including IgA CSR in PPs and the formation of Tregs [469], supporting a local homeostatic role for these cells in IgA production. Indeed, TGF-β production is abundant within the LP from many cell types [42], [465]. Intestinal Tregs are reported to support gut IgA maintenance within the gut LP through production of TGF-β [470], although their contribution to IgA CSR itself is not known.

Therefore, several immune cells can contribute to the maintenance of intestinal plasma cells through the secretion of B cell survival factors. However, whether these are sufficient to drive LP-resident CSR is unknown.

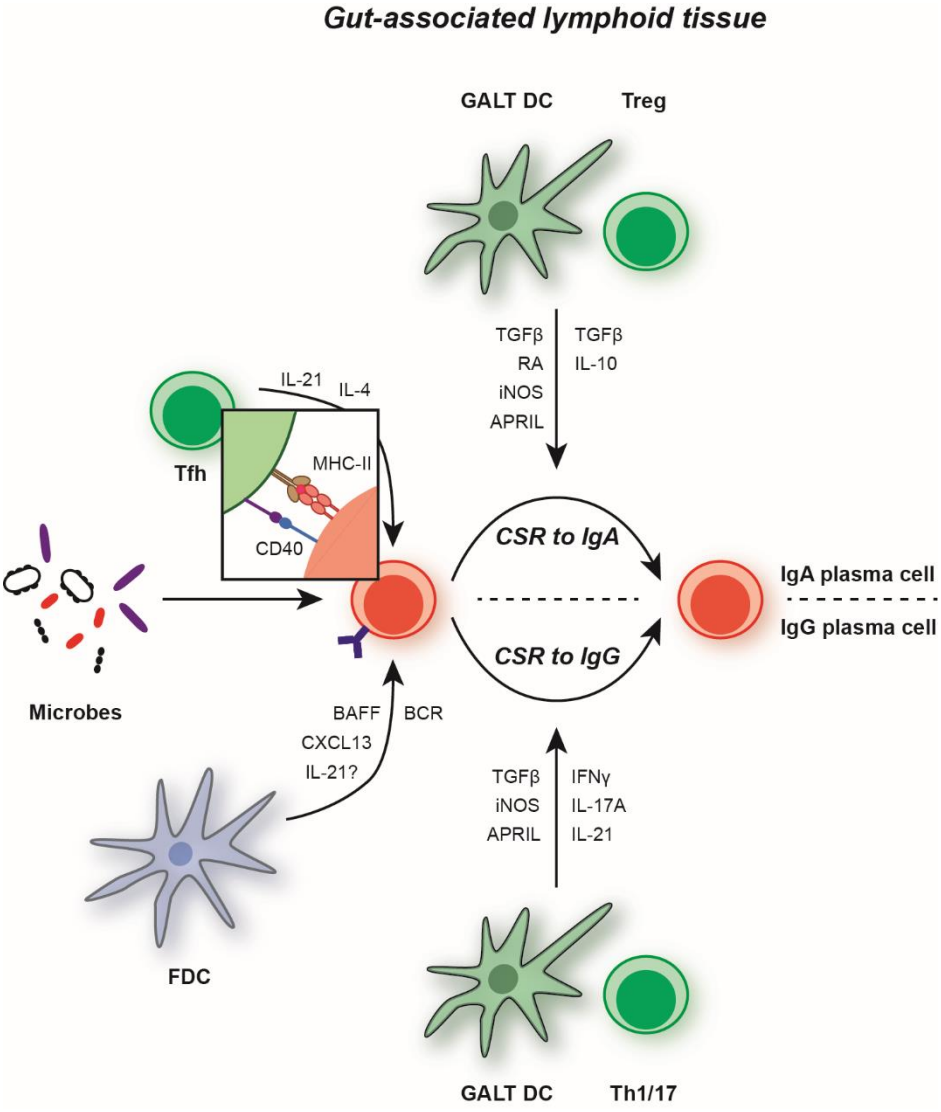

Figure 1.11. Known and hypothesised mechanisms of Ig CSR in GALT. Primary signals initiate B cell activation and the induction of CSR, such as BCR stimulation, BAFF and APRIL, or TLR ligation. CSR to IgA and gut homing via CCR9 and α4β7 is critically dependent on secondary signals from GALT-resident DCs and T cells, including TGFβ, iNOS, and RtA. Th1/17 and DC signals are known to regulate IgG class-switching in mice, including IL-17A, IFNγ, and IL21. How these signals may contribute to IgG class-switching in GALT in unknown.

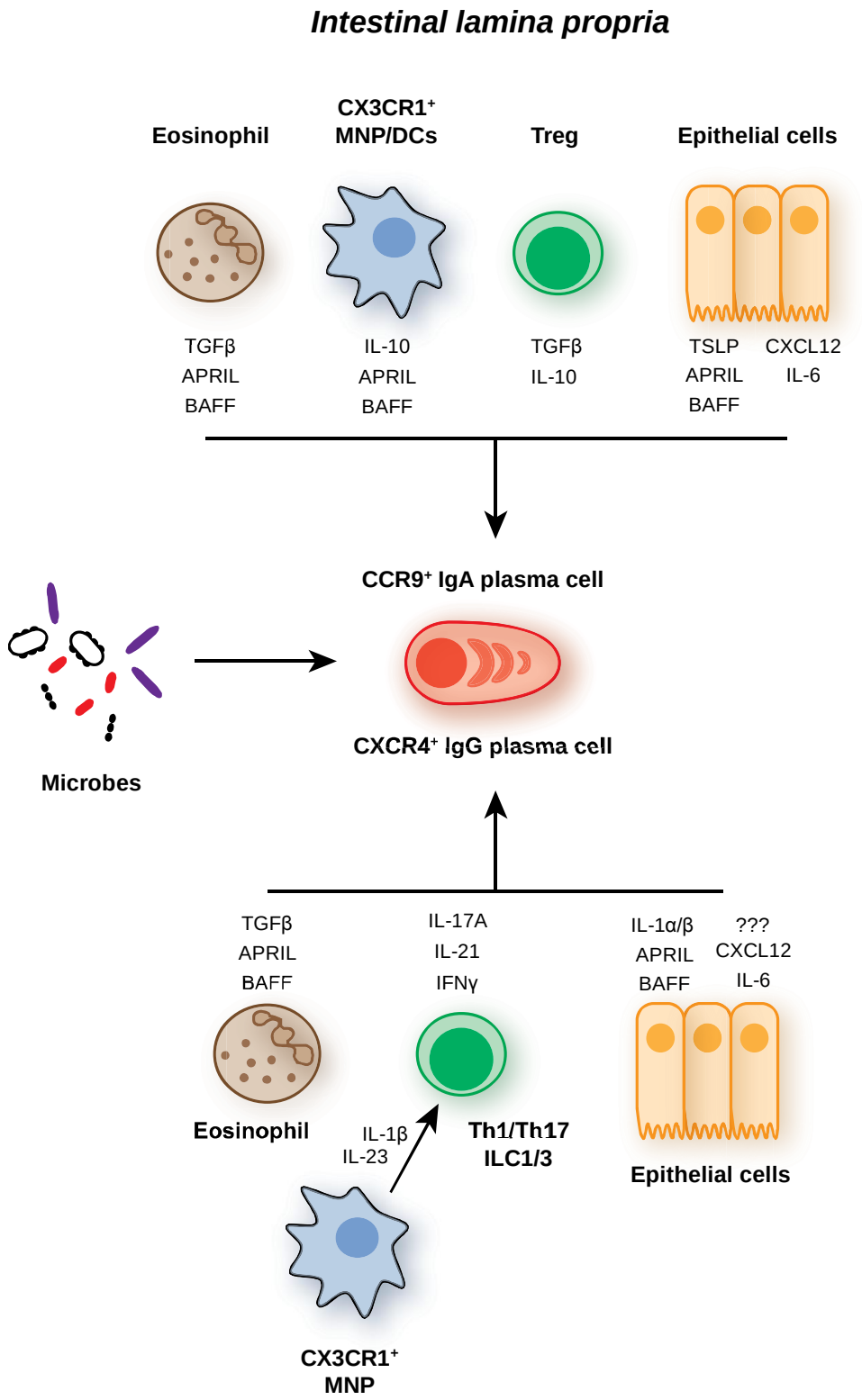

Figure 1.12. Plasma cell niches in the lamina propria. TGFβ, IL-10, BAFF and APRIL production are abundant in the GI tract, and may be derived from a variety of immune cells. Epithelial cell-derived cytokines may also promote IgA CSR in the LP. Alternatively, these factors may promote plasma cell survival within the LP. Numerous inflammatory networks may contribute to TI IgG generation, plasma cell survival, or the induction of glycosylation programmes within the inflamed lamina propria in IBD, including Th1/17 activation, and epithelium-derived cytokines.

1.7.3. IgG in intestinal inflammation

While IgA is the predominant Ig isotype at rest, increasing evidence points towards a critical role for IgG antibodies within the GI tract in the defence against infection, during intestinal immune development, and in chronic inflammatory disorders, such as IBD [471].

1.7.3.1. IgG in gastrointestinal infection

Murine models of infections have identified a role for B cells in gastrointestinal immunity. An IgA/IgM-independent role for B cells in the clearance of *C. rodentium* was shown to be dependent on IgG [283], [284], [472], with antibodies shown to target virulence factors essential for pathogen entry [473], [474].

Several studies in non-human primates have also identified roles for IgG in anti-microbial mucosal immunity. Passive transfer of serum rotavirus-specific IgG has been shown to delay rotavirus infection in naïve pigtailed macaques [475], while anti-HIV IgG administration to rhesus macaques can prevent viral transmission [476].

1.7.3.2. IgG in intestinal immune development

The acquisition of maternally-derived IgG antibodies *in utero* and through breast milk provides neonates with an important source of immunity prior to the development of the host immune system. Recent studies in mice have demonstrated that TI, TLR-dependent anti-commensal IgG generation forms part of normal adult immunity, and that this IgG is essential for the maintenance of host-commensal symbiotic relationship and protection from systemic microbial infection [477]–[479]. In the absence of maternally-derived IgG antibodies, neonates exhibit exacerbated mucosal Tfh responses and GC B cell activation [477], [480], as well as altered tissue-resident immune cell subsets, including reductions in ILCs and macrophages [479]. Furthermore, homeostatic anti-Gram-negative IgG has been shown to confer protection against systemic pathogenic challenge by targeting conserved antigens on pathogens [478]. This humoral response is plastic, as anti-commensal IgG antibodies have been shown to compensate for defects in innate immunity for the maintenance of host-commensal symbiosis: defective TLR signalling or oxidative burst production resulted in enhanced anti-commensal IgG titres that maintain mutualism *in vivo* [481]. As such, IgG-mediated anti-commensal responses represent an essential and plastic mechanism required to maintain host-microbe mutualism and mediate systemic pathogen immunity.

Anti-commensal IgG antibodies and plasma cells are also present in humans [240], and similarly to IgA, have been shown to be generated locally within GALT and MLNs, challenging the assumption that anti-commensal IgG is a phenomenon arising from systemic dissemination of commensal antigens [477].

While encouraging as to the key functional role of anti-commensal IgG antibodies in immunity, the mechanisms by which IgG operates have not been investigated. Indeed, how anti-commensal IgG responses are regulated, how they may contribute to dysbiosis, and their mechanistic contribution to inflammatory conditions remains largely unexplored.

1.7.3.3. IgG in IBD

As previously mentioned, one of the robust associations with UC susceptibility is a SNP in human FcγRIIA that enhances IgG binding. In addition to historical observations of IgG-producing plasma cells in the inflamed mucosa of IBD patients [427], this supports the hypothesis that detrimental local IgG generation may contribute to inflammation via the induction of FcγR signalling pathways.

In line with this, Blumberg and colleagues demonstrated that anti-flagellin IgG is generated following exposure of WT mice to the colitogen DSS, and passive transfer of anti-flagellin IgG was detrimental in colitis [482]. Mice exhibit enhanced weight loss and pathology scores, but the mechanism of this enhancement was not described. Furthermore, how the character of this IgG changes with regard to homeostatic anti-commensal IgG is not discussed. However, this supports work by several groups that demonstrated an increase in mucosal IgG^+ plasma cells in IBD [241], [430].

In summary, recent seminal studies have demonstrated that anti-commensal IgG is a normal facet of adult immunity during homeostatic conditions that contributes to mutualism in the gut. However, IgG responses are exacerbated in colitis and can contribute to detrimental inflammation, although the mechanisms by which this occurs are not understood.

1.8. The intestinal macrophage system

Macrophages play a central role in tissue homeostasis and inflammation, with increasingly appreciated non-immune roles in tissue function [483]–[485]. However, through their voracious phagocytic capacity and ability to secrete both pro- and anti-inflammatory cytokines and chemokines, they have essential roles in the coordination of immunity within the gut and in shaping of systemic responses.

1.8.1. Identification

The distinction between tissue-resident DCs and macrophages, which both belong to the population of mononuclear phagocytes (MNPs), has often proved confusing, given their shared overlap of several cell surface markers, including CD11c, CX3CR1, and MHC-II. This is further compounded by the monocyte-derived origin of certain DC subsets.

The recent demonstration that FcγRI is unique to intestinal macrophages has helped to resolve this issue [68]. Indeed, this study demonstrated that as Ly6C^{hi} monocytes enter the LP, these cells mature by the stepwise acquisition of MHC-II, loss of Ly6C expression, and the upregulation of F4/80 and FcγRI, bone fide macrophage markers, as well as CX3CR1 and CD11c. This allows for a so-called waterfall gating strategy within the CD11b^{+} CX3CR1^{+} MNP population based on Ly6C and MHC-II, which will be used throughout this thesis (Fig. 1.13). Generally, we have dissected this waterfall into three subpopulations:

1. CX3CR1^{int} Ly6C^{hi} MHC-II^{-} monocytes
2. CX3CR1^{int} Ly6C^{+} MHC-II^{+} monocytes/immature macrophages
3. CX3CR1^{hi} Ly6C^{lo} MHC-II^{hi} macrophages, which also express F4/80 and CD11c.

During inflammation, monocytes give rise to a secondary macrophage population, characterised by inflammatory cytokine production [82]. This can be distinguished by lower CX3CR1 and F4/80 expression compared to tissue-resident macrophages. However, in general, this distinction has not been made here, and macrophages are analysed as a single population.

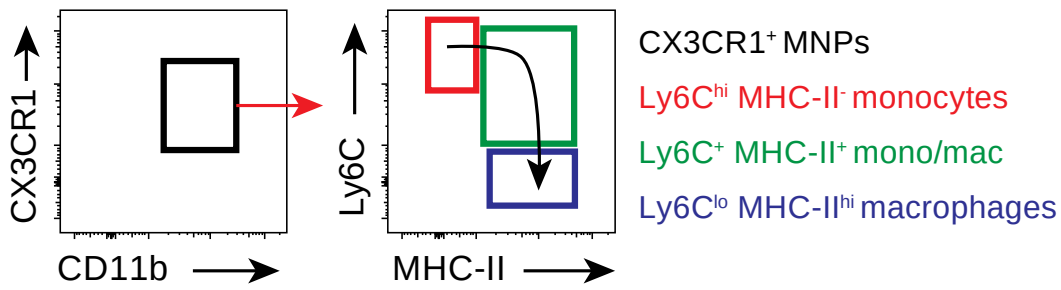

Figure 1.13. Waterfall gating strategy of MNP subsets by flow cytometry. MNPs are identified as CD11b^{+} CX3CR1^{+} cells within the LP. MNPs are then subdivided into monocytes (red), MHC-II^{+} monocytes/immature macrophages (green), and macrophages (blue) based on expression of Ly6C and MHC-II.

Despite this classification, it seems likely that both macrophages and DCs contribute in complementary ways to inflammation. For example, although classically associated with antigen presentation, CD103^{+} DCs can also contribute to flagellin-induced IL-23 production, while macrophage MHC-II expression demonstrated antigen presentation capacity by these cells also [486].

1.8.2. Development

Elegant studies by Mowat and colleagues demonstrated that, while neonatal intestinal macrophages are derived from embryonic precursors cells that seeded the intestinal mucosa and proliferated *in situ*, adult intestinal macrophages are derived from continuous CCR2-dependent Ly6C^{hi} monocyte recruitment in response to the microbiota [280]. This is distinct from other tissue-resident macrophages, such as microglia and Kupffer cells, which are shown to be derived from yolk-sac progenitors and self-renew throughout life [487].

Environmental and host factors condition monocyte maturation within the LP to avoid overt inflammatory responses, although this area remains poorly understood [82], [488]. While intestinal macrophage numbers are suggested to be generally unaltered in GF mice [489], commensal microbes influence macrophage activation within the GI tract [490], [491]. Indeed, SCFAs, particularly butyrate, derived from the microbiota have been implicated in the appropriate maturation of intestinal macrophage and microglia [492], [493]. Butyrate acts to suppress inflammatory IL-6 and IL-12 production, likely contributing to the maintenance of mutualism. GF mice exhibit also reduced bone marrow myelopoiesis and susceptibility to systemic bacterial challenge, with implications for the *de novo* generation of monocyte precursors and the continuous seeding of the GI tract [494], [495].

Intrinsic IL-10R signalling appears to be essential for the restriction of intestinal inflammation through the maintenance of an anti-inflammatory macrophage phenotype [425]. Recently, BMDMs were shown to adopt a tissue-resident phenotype within the peritoneum in response to RtA [496]. Given the pleiotropic roles of RtA in the maintenance of the symbiotic niche, it is may be that a similar role exists in the gut.

In summary, gut-resident macrophages are derived from circulating monocyte precursors throughout life and are subject to regulation by the local cytokines and the microbiome for appropriate maturation and activity.

1.8.3. Intestinal macrophages in homeostasis

Within the GI tract, macrophages are continuously exposed to microbial stimuli and play a central role as key guardians of the homeostatic milieu. They are characterised by their prominent phagocytic capacity and an anti-inflammatory phenotype, being hypo-responsive to TLR stimulation and potent producers of IL-10 [497], [498]. Some of these functions are summarised in Figure 1.14.

1.8.3.1. Scavenging

One of the primary homeostatic functions of intestinal macrophages is scavenging of self and foreign antigens, such as apoptotic cells and bacteria, respectively. To this end, these cells

express an array of cell surface receptors, including TLRs, C-type lectin receptors, Nod-like receptors, and FcγRs [3]. Despite this, intestinal macrophages are relatively anergic to microbial stimuli [499], [500].

These phagocytic functions are essential in the gut, given the continuous turnover of the intestinal epithelium and challenge by microbial products. Defective apoptotic clearance by macrophages and epithelial cells has been shown to drive autoimmunity and colitis [271], [501]–[503]. Furthermore, as well as promoting the clearance of DAMPs and preventing the emergence of self-reactive adaptive immunity, apoptotic cell internalisation also imprints macrophages with inflammation-suppressive transcriptional signatures and the production of TGFβ and PGE2 [500], [504], [505].

1.8.3.2. Cytokine production

Gut macrophages are characterised by their elevated production of IL-10 and TNF, as well as CXCL chemokines involved in neutrophil recruitment into tissues [82]. Macrophage-derived IL-10 is required to dampen IL-23-mediated inflammation and likely contributes to autocrine suppression, although these functions may be redundant with other IL-10-producing cells [425], [498]. However, via their secretion of IL-10, RtA, and exogenous TGFβ, intestinal macrophages can also induce Tregs *in vitro* [497]. Furthermore, homeostatic IL-1β secretion by intestinal macrophages is required for the priming of tolerogenic Treg-inducing DCs through IL-1β-dependent GM-CSF secretion by ILC3s [506].

Given their close proximity to the epithelium, macrophage-derived secreted factors are also suggested to contribute to epithelial wound repair in colitis, including COX-2-derived prostaglandins and Wnt family proteins [490].

As well as in the LP, macrophages are also present between the circular and longitudinal muscle layers of the GI tract. These muscularis macrophages are skewed towards anti-inflammatory function, with elevated levels of IL-10 and arginase, and lower production of IL-12p40 and NOS2. Furthermore, these cells can response directly to neurotransmitters, which boost the anti-inflammatory function of these cells *in vivo* [484].

1.8.3.3. Antigen presentation

Other than the maintenance of Tregs within the LP itself, macrophages play dual roles in antigen presentation to T cells.

Firstly, $CX3CR1^{+}$ MNPs within the intestinal LP have been shown to acquire antigens directly from the lumen via intraepithelial projections and transfer antigen to migratory DCs for transport to the MLN [507]–[509]. Furthermore, while less efficient than DC subsets, evidence suggests

that CX3CR1^{+} macrophages can transport internalised antigens directly to draining LNs through intrinsic expression of CCR7 and MHC-II [82], [497].

1.8.3.4. Physiology

Recent studies have demonstrated roles for macrophages in intestinal physiology. Macrophages located within the intestinal muscularis externa regulate the peristaltic activity of the colon through the secretion of bone morphogenic protein 2 (BMP2) in response to microbial stimuli [483]. BMP2 regulates enteric neuron activity, which subsequently maintain macrophages through the production of macrophage-colony stimulating factor (M-CSF).

In summary, intestinal macrophages have critical roles in intestinal homeostasis, from the engulfment of apoptotic cells, the production of anti-inflammatory mediators, the induction and maintenance of Tregs, and the regulation of intestinal motility.

1.8.4. Intestinal macrophages in intestinal inflammation

1.8.4.1. Development

Macrophage infiltrates are a common feature of inflammation and pathology [3]. As intestinal macrophages under both inflammatory and homeostatic conditions are derived from blood monocytes, the MNP dynamic differs significantly during colitis. In response to DSS administration, Ly6C^{hi} monocytes are redirected from an anti-inflammatory phenotype to one of elevated inflammatory cytokine production [510]. Indeed, these cells now adopt a CX3CR1^{int} MHC-II^{hi} phenotype, rather than CX3CR1^{hi}, and come to dominate the LP. Unsurprisingly, therefore, CX3CR1^{int} macrophages are principle mediators of tissue inflammation and skewing the MNP-driven response to one of leukocyte recruitment and local immune cell activation [82].

1.8.4.2. Inflammatory cytokine production

Macrophages are identified as a major source of TNF in the inflamed LP. TNF-producing macrophages are observed close to the epithelium in UC, while they are distributed throughout the mucosa in CD [511].

Furthermore, several murine models have implicated CX3CR1^{+} MNPs, particularly monocytes and Ly6C^{+} CX3CR1^{int} immature macrophages, as essential mediators of inflammation via their production of IL-23 and IL-1β, as previously mentioned [82], [295], [510]. IL-23 production by CD11c^{+} MNPs was also shown to be key to driving *H. hepaticus*-induced colitis: in the absence of CD11c-specific IL-23, IFN-γ and IL-17A responses were severely inhibited and improved disease progression [295]. Furthermore, IL-1β, IL-23 and TL1A derived from CX3CR1^{+} macrophages supports colitis-associated IL-22 and GM-CSF production by ILC3s *in vivo*,

which may contribute to the pathogenic response [291], [292], [360], while IL-1β-mediated Th17 accumulation is associated with chronic *H. hepaticus*-induced colitis [296].

An important example of type 17-mediated immune responses in the GI tract regulated by CX3CR1^{+} macrophages is the response to segmented filamentous bacteria (SFB) in mice. These bacteria are important inducers of intestinal Th17 cells, enhancing defence against *C. rodentium* infection [310]. An elegant study by Littman and colleagues demonstrated that SFB induced IL-23-driven IL-22 production by ILC3s and the subsequent induction of local IL-17A production by Th17 cells in response to SAA1/2 secreted by the intestinal epithelium [512]. Therefore, macrophages coordinate ILC3 and Th17 cells for distinct aspects of intestinal immunity.

Furthermore, SFB-driven IL-17 responses are associated with extra-intestinal pathologies. For example, SFB-induced Th17 cells exacerbated arthritis development in the murine K/BxN model via an IL-17-dependent mechanism [513]. Specifically, IL-17 supported GC formation and autoantibody generation. Similarly, SFB promoted EAE via Th17 cell induction [514], while trafficking of intestinal IL-17A-producing γδ T cells to the leptomeninges is associated with increased ischemic brain injury in a murine model of stroke [515].

Given the demonstration that CD103^{+} DCs also express IL-23 in response to flagellin, it seems likely that these functions are shared with other MNP subsets in a context-dependent manner [486].

Tissue-resident macrophages not only drive inflammation through the production of inflammatory cytokines, they may also directly orchestrate immune cell recruitment via the production of chemokines. Tissue-resident CX3CR1^{hi} macrophages directly secrete CCL2 and CCL8 in murine models of DSS-induced colitis, driving monocyte recruitment to the inflamed mucosa. Furthermore, these cells also express high levels of neutrophil-recruiting chemokines, including CXCL1 and CXCL2 [82], [516]. While this is likely to contribute to mucosal immune defence, it can potentially drive sustained detrimental inflammation if unchecked.

1.8.4.3. Antigen presentation

As well as the induction of Tregs, FcγRI^{+} macrophages are able to directly induce pathogenic Th1 responses within MLNs during colitis [68]. Whether these cells arise from migration from the LP is not clear, but is consistent with the upregulation of CCR7 on gut-resident inflammatory macrophages [82].

Therefore, inflammation alters the developmental profile of gut-infiltrating monocytes from one of IL-10 production and Treg induction to one of inflammatory cytokine production,

characterised by IL-1β and IL-23 secretion, the induction of Th1 and Th17 responses, and the orchestration of tissue damage.

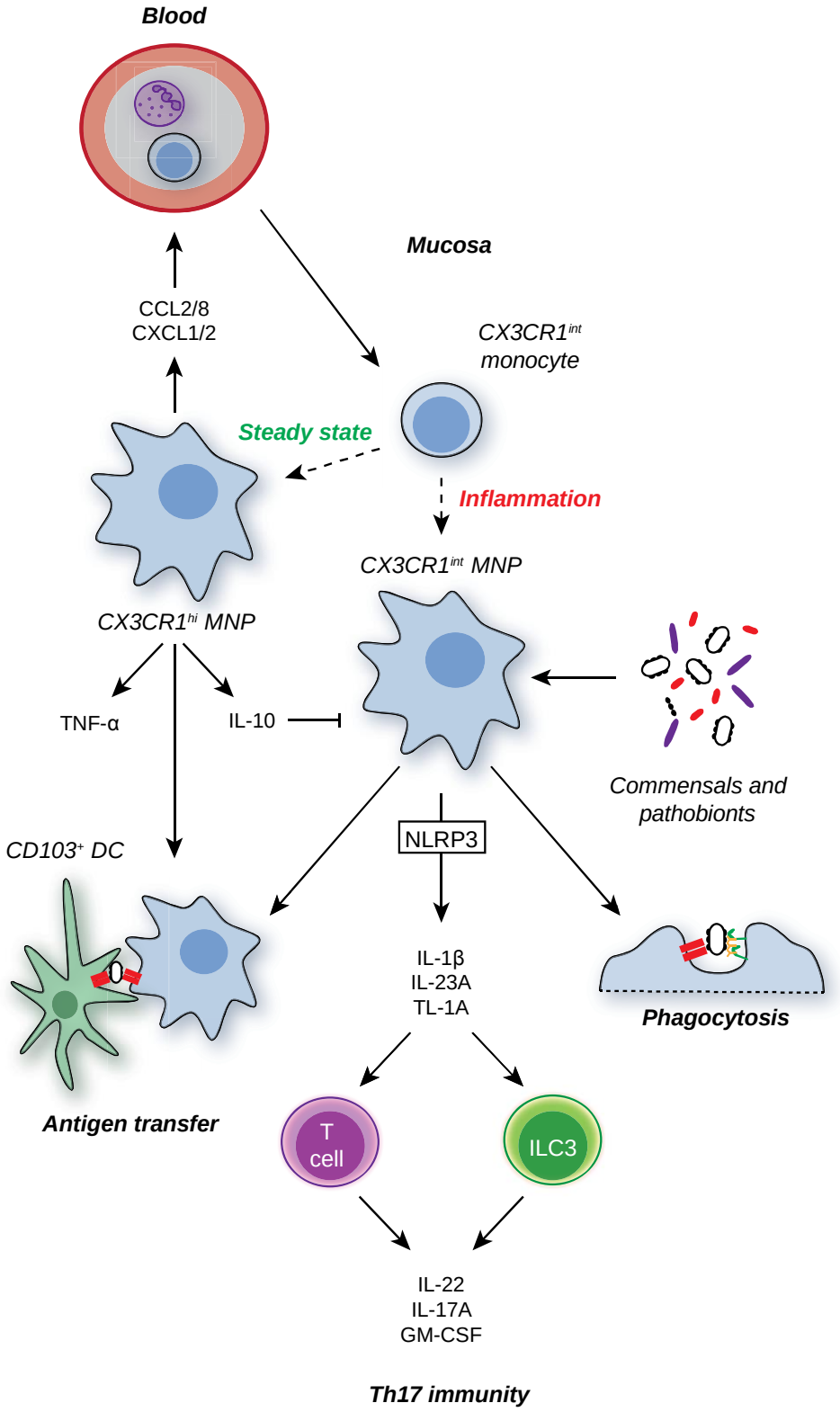

Figure 1.14. Macrophage functions in homeostasis and inflammation in the GI tract. Macrophages are continuously derived from blood monocytes that seed the gut. These monocytes mature into IL-10-producing phagocytic macrophages under homeostatic conditions. Under inflammatory conditions, tissue-resident macrophages produce pro-inflammatory cytokines, such as IL-1β and IL-23, and chemokines, including CXCL1 and 2 (mice) and CXCL8 (humans) that contribute to inflammatory through several mechanisms.

1.8.5. The role of FcγRs on intestinal macrophages

FcγR-mediated effector functions have been shown to be essential in several circumstances of mucosal anti-microbial immunity.

One Fc receptor that has received growing attention as a vehicle for IgG-mediated immunity is the FcRn, driven by pioneering work by Blumberg and colleagues. Classically associated with its role in the bidirectional transport of IgG across epithelial surfaces [517], FcRn is also expressed by intestinal immune cells, particularly myeloid cells, whereby it increases the half-life of monomeric IgG by preventing its degradation. Furthermore, FcRn promotes antigen presentation of immune complexed-antigens via MHC class I and II [518], [519].

FcRn binding is required for IgG-mediated enhancement of DSS-induced colitis [482]. Furthermore, FcRn binding enhances the anti-microbial activity of IgG in several models, including HIV and SIV challenge [520], while cancer immunity by $CD8^+$ T cells is enhanced by FcRn-mediated IL-12 production by DCs [521], [522].

Other than FcRn, anti-microbial activities ascribed to IgG in the defence against enteropathogens, such as *C. rodentium*, is dependent on binding to canonical FcγRs [523]. In the absence of the common γ-chain, *Fcer1g*, mice readily succumb to *C. rodentium* challenge, exhibit enhanced tissue damage, reduced DC maturation, reduced TNF production, and impaired macrophage bacterium phagocytosis. Similarly, *in vitro* studies by Uo et al. demonstrated that stimulation of $CD14^+$ macrophages with opsonised bacteria induced elevated expression of IL-1β, TNF and TL1A versus bacteria alone, with little effect on IL-12 and IL-23 [430].

Therefore, while the dissection of the roles of FcγR signalling on intestinal macrophages is in its infancy, studies support a role for local IgG and FcγR engagement in inflammation and immunity in the GI tract. Furthermore, the study by Uo et al. raises the possibility that macrophage-intrinsic FcγR signalling may drive colitis through the production of several key IBD-associated cytokines. In addition, it seems likely that FcγR signalling in intestinal macrophages contributes to anti-inflammatory mechanisms, such as IL-10 production. However, how these observations translate *in vivo* to murine models and human disease is still unknown.

1.9. Innate lymphoid cells

1.9.1. Overview

ILCs are a recently identified class of immune cells that are enriched at mucosal sites, such as the GI and respiratory tracts [12], [524]. The subject of intense research in the last half-decade, ILCs are increasingly appreciated to play diverse and critical roles in the maintenance of tissue homeostasis and in driving and shaping inflammatory responses, primarily through their elevated production of cytokines [12], [524]–[530].

1.9.2. ILC subsets

Despite initial confusion as to the nature and relationship between described subsets, ILC classification has improved with time, and is likely to diversify in future as novel functions are discovered [531]. Strikingly, ILCs subsets mirror those seen for T cells. NK cells represent the innate cytotoxic equivalent of $CD8^+$ T cells, and will not be discussed in detail here, whereas non-cytotoxic *helper* ILCs resemble Th cells. All helper ILCs, hereafter referred to simply as ILCs, express IL-2Rα and IL-7Rα, but unlike T cells and B cells, they do not express somatically-rearranged antigen-specific receptors [528].

There are three major groups of ILCs, classified based on cytokine profile and TF dependence (Fig. 1.15). Group 1 ILCs (ILC1s) produce IFNγ and TNF in response to IL-12 and IL-18, and are implicated in immunity against intracellular bacteria and viruses. Despite their similarities to NK cells, ILC1s do not produce granzymes or perforin and their development is dependent on T-bet, unlike Eomes-dependent NK cells [532]. Group 2 ILCs (ILC2s) produce Th2-associated cytokines, including IL-5, IL-9 and IL-13, as well as amphiregulin, in response to the epithelial-derived cytokines IL-33, IL-25, and TSLP. As such, they drive allergic inflammation, anti-helminth immunity and tissue repair [533], [534]. These cells are dependent on RORα, GATA3, TCF1, and Bcl11b [535]–[540]. Via their expression of MHC class II, ILC2 can also licence adaptive immunity via antigen presentation and DC priming [541]–[543]. Finally, group 3 ILCs produce type 17 cytokines, including IL-17A, IL-17F, IL-22, GM-CSF and TNF, particularly in response to IL-23 and IL-1β [292], [296], [359], [544], [545]. All ILC3s are dependent on the TF RORγt [546], but can be divided into three subsets, based on T-bet and NKp46 expression, each with distinct functions [547]:

1. T-bet^+ $NKp46^+$ ILC3s, also known as NK cell receptor-positive (NCR^+) ILC3s,
2. T-bet^+ $NKp46^-$ ILC3s, and
3. T-bet^- $NKp46^-$ $CCR6^+$ $CD4^{+/-}$ ILC3s, also known as lymphoid tissue-inducer (LTi) cells.

Mature $NKp46^+$ ILC3s reside primarily in the intestinal mucosa and, as well as RORγt and T-bet, require aryl hydrocarbon receptor (AHR), TCF-1 and the microbiota for their development

[547]–[549]. Here, these cells primarily regulate protective barrier function via IL-22 production, as shall be discussed in detail.

LTi cells represent a distinct ILC3 subset that develops partly independently of other helper ILC subsets [550]. Originally identified as cells required for the development of lymphoid tissues via their production of lymphotoxin-α1β2 (Ltα1β1) [546], [551], these cells predominantly reside within SLOs and are also associated with production of IL-22 and IL-17A, and MHC class II-dependent T cell interactions [552]–[554]. While less dependent on AHR, a seminal study demonstrated that RtA derived from maternal vitamin A and cell-autonomous RtA signalling is required for transcription of the *Rorc* locus and LTi development [555].

Other than developmentally programmed phenotypes, tissue-residency imparts unique ILC functionality. For example, small intestinal ILCs across several subsets commonly expressed unique transcriptional traits distinct from corresponding subsets in distant tissues [556], [557].

Therefore, ILCs represent a unique subset of innate immune cells that provide complementary and unique functions to T cells, and likely act to bridge the temporal gap between activation of innate immunity and the development of antigen-specific responses.

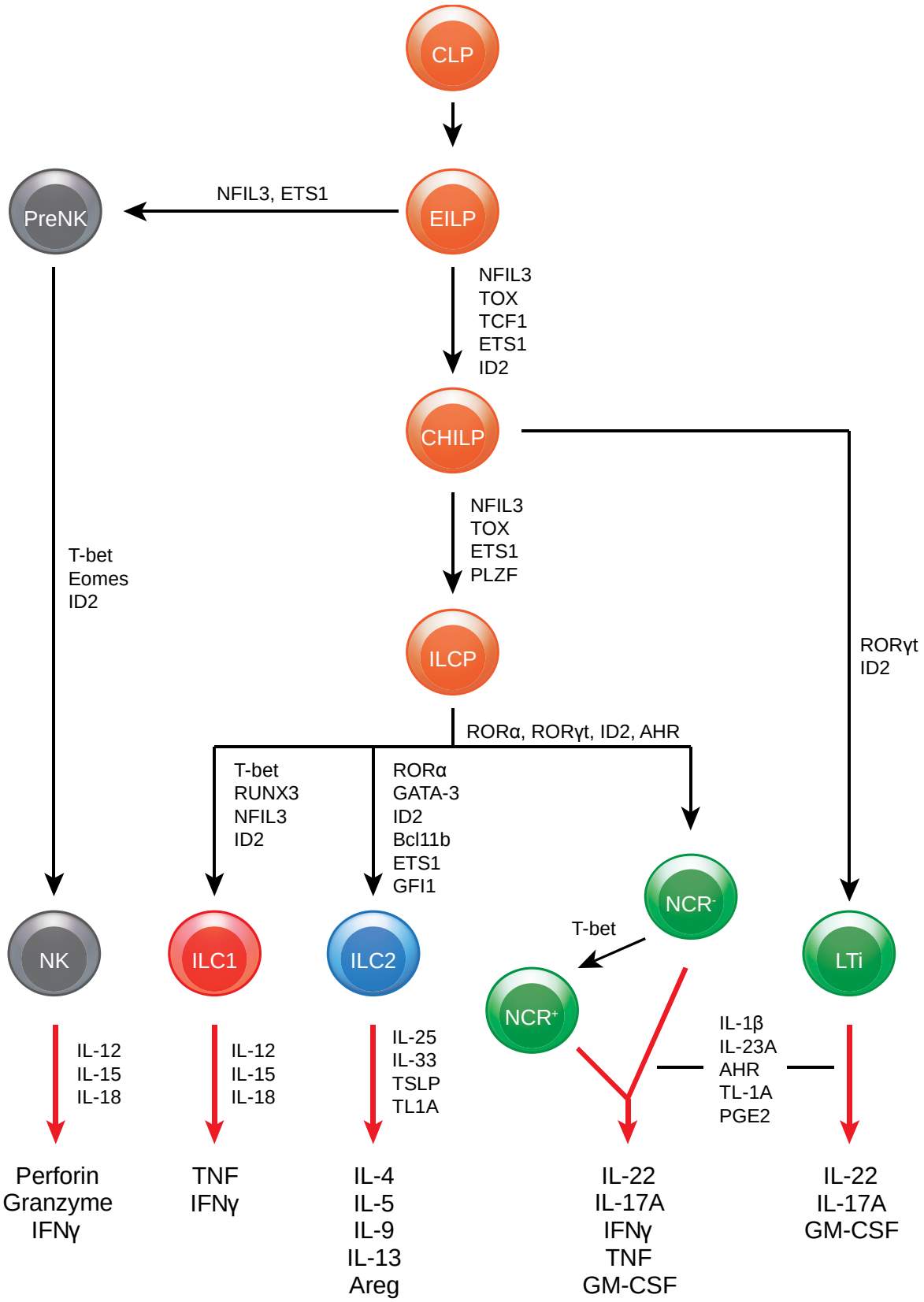

Figure 1.15. Development and function of cytotoxic and helper ILC subsets. Cytotoxic NK cells develop from the EILP in a Eomes and T-bet-dependent manner. Helper ILCs are derived from the ILCP, apart from LTi cells, such differentiate from the CHILP. Transcription factor and cytokine-dependence of ILC subsets is demonstrated on the right-hand side of each arrow. Characteristic cytokines produced by each ILC subset are listed.

1.9.3. Development

Many multipotent progenitors have been identified that are able to generate ILCs (Fig. 1.15). In accordance with their lymphocyte origin, all ILCs develop from CLPs: CLPs isolated from the fetal liver and bone marrow being able to generate ILCs *in vitro* and *in vivo* [558], [559]. ILC development then proceeds through a series of progenitors with increasingly restricted potential, firstly through the loss of T and B cell developmental potential. These early innate lymphoid progenitors (EILPs) are NFIL3, TCF-1 and TOX dependent [550], [560], [561]. An ID2-dependent common helper ILC progenitor (CHILP) then gives rise to all helper ILCs, but not NK cells [562]–[564]. While lacking significant expression of GATA-3, T-bet, RORγt or RORα, this heterogeneous population includes a subset that express PLZF and PD-1 [559], [565]. $PLZF^+$ CHILPs are more differentiated, with GATA-3 expression and an inability to form LTis, and are called helper ILC progenitors (ILCP). Indeed, although classically associated with mature ILC2s, GATA-3 expression by ILCPs is necessary for the development of all ILCs [566], [567]. In humans, circulating and tissue ILCPs have been identified that are homologous to murine ILCPs, and exhibit a poised chromatin landscape with multi-ILC lineage potential [568].

Therefore, although in its relative infancy, ILC developmental programmes are being uncovered that demonstrate significant similarity between helper ILC, and identifying NK cells and, to a lesser extent, LTis, as developmentally restricted populations from other ILCs.

1.9.4. ILC3s in the GI tract

The GI tract represents one of the major sites of ILC colonisation, with a particular abundance and functional significance attributed to ILC3s, which will be the major subject of discussion here (Fig. 1.16) [569].

1.9.4.1. ILC3s in epithelial barrier integrity

Through their production of IL-22, ILC3s play a critical role in the reinforcement of the intestinal epithelial barrier. This is required for protection against the dissemination of opportunistic commensal species and pathogens, and has been well described in the literature.

Early studies demonstrated that *C. rodentium* infection induced innate intestinal IL-22 production that was dependent on IL-23 and the microbiota [285], [287]. Although initially ascribed to DCs, RORγt-dependent ILC3s were later identified as the predominant source of IL-23-induced IL-22 in both mice and humans [292], [552], [570]–[572].

IL-22R signalling induces STAT3 activation in IECs, driving the production of Reg family anti-microbial peptides and mucus that regulate microbial invasion [512], [545], [573], [574]. IL-22 and lymphotoxin-dependent IEC fucosylation was also shown to be required for protection

against *Salmonella typhimurium* infection [575]. Hanash and others have demonstrated roles for IL-22 in the protection and turnover of intestinal $Lgr5^+$ stem cells in several models of epithelial damage and in organoid systems [576]–[579]. ILC3s are maintained in close proximity to the epithelium via a CXCL16/CXCR6 axis, whereby gut-resident $CX3CR1^+$ MNPs secrete this chemokine in response to microbial stimulation [580]. Therefore, the ILC3s are well placed to effect widespread control over the intestinal epithelium through secretion of IL-22.

In addition to the well-described roles of IL-23 and IL-1β in the activation of ILC3s, several other stimuli have been uncovered that regulate IL-22 production. Systemic LPS administration was shown to promote PGE2 production and the activation of intestinal IL-22 production via PTGER4 signalling on ILC3s [544]. Furthermore, other MNP-derived cytokines, such as TL1A [291], and CD1d-mediated cross-talk with invariant NK T cells have also been shown to promote IL-22 production by these cells [581]. Finally, glial cell-derived neurotrophic factor receptor, produced in response to TLR and alarmin stimulation, could induce IL-22 and promote protection against DSS-induced inflammation, highlighting a neuroimmune feature of ILC3 functional regulation [582].

Given its largely beneficial roles, a promising avenue in IBD therapy would be to specifically target damaging Th17 immune responses, while maintaining IL-22 production by ILC3s to repair damage to the epithelium. To this end, Wither et al. were able to transiently suppress Th17 cells during *C. rodentium* infection using a RORγt inhibitor, while preserving ILC3 activity. This arises due to the differential requirements of these two cell populations for continuous RORγt signalling, and resulted in reduced disease severity [289].

However, care must be taken in extrapolating the effects of IL-22, as not all functions of ILC3s on the intestinal epithelial barrier are beneficial to the host. IL-22 was recently demonstrated to induce IL-18 production by IECs directly, which, while protective in *C. rodentium* infection, resulted in enhanced IFNγ-driven pathology following *T. gondii* challenge [583]. In addition to the well-documented roles of IL-22, ILC3s are also a source of IL-17A [552]. ILC-derived IL-17A exacerbated intestinal cancer progression in response to hyperactivated IL-23R signalling, although IL-17A alone was not sufficient for tumorigenesis [584].

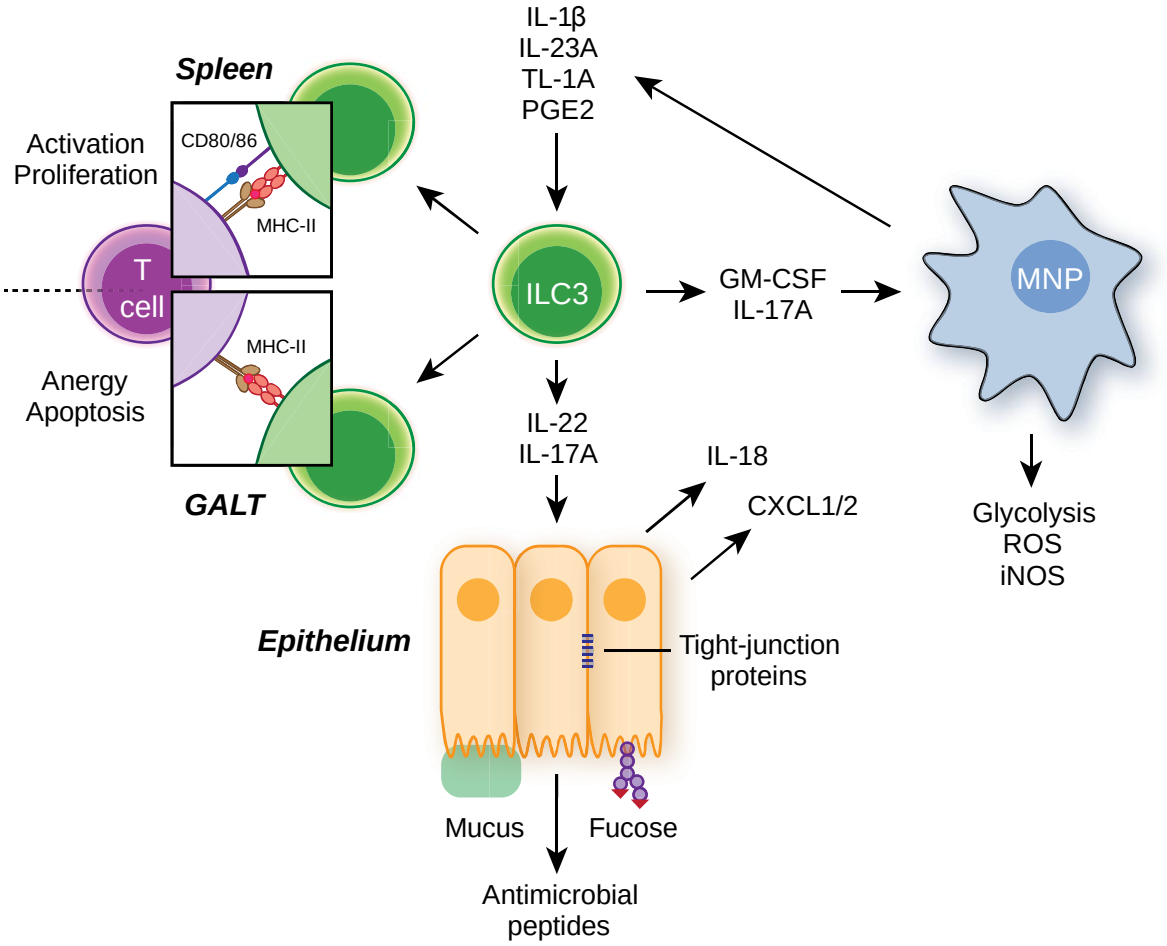

Figure 1.16. Functions of ILC3s within the GI tract. Via their production of IL-22, IL-17A, and GM-CSF, ILC3s potentially can influence immune responses in epithelial cells and macrophages. Furthermore, via expression of MHC-II, ILC3s can induce anergy and apoptosis or activation of $CD4^+$ T cells, depending on co-expression of CD80 and CD86.

1.9.4.2. ILC3s in anti-microbial immunity

The roles of ILC3s in regulating anti-microbial immunity have received relatively little attention compared to effects on the epithelium. However, emerging data suggests that ILC3s play critical roles in the coordination of immune cells at mucosal sites.

A recent study by Xiong et al. demonstrated that monocyte-derived TNF promoted ILC3 recruitment to the lung following *Klebsiella pneumoniae* infection. This initiated a positive feedback loop that resulted in IL-17A-mediated enhancement of monocyte anti-microbial immunity [585]. Whether these observations translate to the intestine is yet to be seen, but directly implicates ILC3s in the regulation of macrophage biology.

ILC3s are also an important source of GM-CSF, a key regulator of tissue immunity. In complementary studies, ILC3-derived GM-CSF was shown to orchestrate monocyte and

granulocyte recruitment to the inflamed LI LP in anti-CD40 and *H. hepaticus* models of colitis [297], [586].

Given studies demonstrating the role of GM-CSF in regulating macrophage glycolysis and cytokine production, ILC3s could directly regulate intestinal macrophage anti-microbial capacity and inflammatory phenotype via regulation of cellular metabolism. However, this remains to be investigated *in vivo*.

1.9.4.3. ILC3s in tolerance

In addition to their roles in cytokine production, ILC3s have also been demonstrated to induce anergy and apoptosis in intestinal anti-commensal $CD4^+$ T cells via their expression of MHC-II in the absence of co-stimulatory molecules [554], [587]. This effect seems to be largely restricted to LN-resident LTi cells, which are well-placed in the T cell paracortex to influence T cell responses [588], although MHC-II expression is widespread amongst ILCs in different tissues. In contrast, splenic and lung ILC subsets have been show to induce T cell division in model antigen systems [543], [589].

As well as via MHC-II, ILCs have also been shown to express other inhibitory molecules *in vivo*, such as PD-L1 [590], a co-inhibitory molecule known to have key roles in the inhibition of Tfh and GC responses [591], and CTLA4, a CD28-binding CD80/86 antagonist [360].

Seminal studies by Mortha et al. demonstrated that microbiota- and IL-1β-dependent ILC3 GM-CSF production was critical for the maintenance of tolerogenic DCs with in the GI tract [506]. Supporting the idea of macrophage-ILC crosstalk, IL-1β production was itself dependent on GM-CSF production.

1.9.4.4. ILC3 regulation within the GI tract

Intestinal ILC3s are heavily dependent on external signals for their survival and activation in the GI tract. These are derived from other tissue-resident immune cells, such as macrophages, but also from external ligands.

One ligand-dependent TF required for ILC3 biology is AHR. AHR binds directly to exogenous ligands, such as the pollutant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and dietary ligands from the Brassicaceae family of vegetables, and endogenous ligands, such as 6-formylindolo[3,2-b] carbazole (FICZ). Inactive cytosolic AHR is part of a complex with 90-kDa heat shock protein (HSP90). Ligand binding triggers a conformational change that allows AHR phosphorylation and its entry into the nucleus, where it interacts with AHR nuclear translocator (ARNT) and other co-activators to bind target genes [592].

AHR has also been shown to be essential for IL-22 production and ILC3 survival [288], [548], with AHR deficiency severely impairing, and exogenous administration of AHR ligands

promoting, ILC3 maintenance and activity in the GI tract. AHR is proposed to drive Notch signalling in ILC3s, key to ILC3 development, as well as synergising with RORγt to drive expression from the *Il22* locus [288], [593]. In the absence of AHR, reduced IL-22 expression allows for the expansion of SFB and the induction of pathogenic Th17 cells [594].

Vitamin A and its metabolite RtA have been shown to have critical roles in intestinal ILC3 biology. LP-resident ILC3s were shown to be severely compromised in number and IL-22-producing capacity in the absence of vitamin A, resulting in enhanced susceptibility to *C. rodentium* infection [569], [595]. As well as development and maintenance, RtA has been suggested to promote ILC3 migration to the gut via induction of CCR9 expression [596]. However, these studies were conducted largely on *in vitro*-generated ILC3s from progenitors in the spleen.

1.9.5. ILC plasticity

Rather than representing terminally-differentiated subsets, it is increasingly clear the ILCs exhibit remarkable plasticity, allowing for their adaptation to the needs of the tissue microenvironment. ILC1s accumulate at inflamed mucosal sites [597]. While migration of ILCs has been suggested in certain circumstances [588], [598], parabiosis experiments have demonstrated that ILC migration is not commonplace [599]. Therefore, ILC subset expansion is at least partially driven by reprogramming of tissue-resident ILCs *in situ.*

Within the GI tract, RORγt$^+$ ILC3s can lose RORγt expression and induce IFNγ production in response to IL-12 and IL-15 (so-called *ex*-ILC3s) [600]. CD-associated CD14$^+$ MNPs promote polarisation of human ILC3s to ILC1s via their production of IL-12, while IL-1β, IL-2, IL-23, and RA induced the reconversion of *ex*-ILC3s (RORγt$^-$ IFNγ$^+$) back into RORγt$^+$ ILC3s capable of producing IL-22 [601]. IL-2 and AHR signalling have also been implicated in promoting the transition to ILC1s and ILC3s, respectively [357], [602].

Similarly, within the lung, IFNγ-producing ILC1s arise from ILC2s in an IL-12-dependent manner in severe chronic obstructive pulmonary disease. This effect could be reversed by eosinophil-derived IL-4 [603]. A separate study demonstrated that IL-1β could also drive an ILC2-to-ILC1 transition by upregulating IL-12Rβ2 and T-bet expression [604]. However, the mechanistic underpinning of ILC plasticity is still largely unknown. In this regard, insights from Th plasticity may inform our understanding, given the similarities between these two classes of cells.

Balanced STAT3 and STAT5 signalling seems to control the emergence of T cell subsets. STAT3 phosphorylation is induced downstream of IL-23, IL-6 and IL-21 receptors, promoting Th17 differentiation *in vitro* and *in vivo*. In contrast, IL-2-mediated STAT5 phosphorylation favours the emergence of Th1 cells. STAT3 signalling was shown to be necessary for IL-22

and IL-17A production by intestinal $CD4^+$ T cells and the control of *C. rodentium* infection [605]. This TF targets several Th17-associated genes, including *Il17a*, *Ahr*, and *Rorc*, and is required for the development of T cell-dependent colitis [606]. In contrast, STAT5 signalling promotes Th1 development via the induction of IL-12Rβ2 and T-bet expression [607]. Furthermore, IL-2 actively inhibits Th17 development via the suppression of IL-6 signalling and interference with STAT3 binding to target genes [608].

Veldhoen et al. demonstrated that AHR promotes Th17 differentiation by limiting the activation of STAT5 and the effects of IL-2 [609]. Furthermore, STAT3 and AHR cooperate to regulate the chromatin landscape of Th17 cells via the induction of Aiolos (encoded by the *IKZF3* gene), an epigenetic modifier that silences the *Il2* locus [610]. Therefore, the ILC3 defects in *Ahr*-deficient mice may result from altered ILC3 maintenance within tissues and augmented transition to alternative ILC states.

Together, these observations support the hypothesis that IL-23R, AHR, and STAT3 signalling contribute to the maintenance of ILC3s via the suppression of ILC1 differentiation programs, inhibition of IL-2 signalling, and the induction of epigenetic modifiers and type 17-associated genes, such as *RORC*.

1.9.6. FcγRs and ILCs

Several lines of evidence suggest that FcγR signalling may regulate ILC function, either directly or indirectly. However, how IgG regulates ILC biology *in vitro* and *in vivo* is not known.

Firstly, several studies support the hypothesis that FcγR signalling on MNPs may drive ILC3 activation through the production of type 17-inducing cytokines, such as IL-1β and TL1A [74], [430], [611], [612]. Secondly, FcγRIII on murine ILC3s has been suggested in transcriptomics analyses and ILC3-like cell lines [613], [614]. Finally, the close relationship to NK cells, which are known to express activating FcγRs in both mice and humans, suggests that FcγR expression may be a function shared between cytotoxic and helper ILC subsets.

However, no functional significance to date has been ascribed to these observations and nothing is known about how FcγR signalling may regulate ILC biology.

1.10. Hypotheses and aims of PhD

The research pursued over the course of this PhD has resulted in three independent areas of interest. The hypotheses and aims governing these sections will be discussed in turn, as presented in the subsequent results chapters. Nonetheless, all chapters share conceptual and experimental relevance, which should be taken into account.

1.10.1. The role of anti-commensal IgG and Fcγ receptors in IBD

Hypotheses:

1. An anti-commensal IgG response is induced in IBD
2. This mucosal IgG engages FcγRs on local and recruited immune cells
3. FcγR signalling on intestinal immune cells drives intestinal inflammation

The aims of this section of the thesis are to:

1. Characterise the IgG response in patients with UC and in a murine model of intestinal inflammation
2. Identify major inflammatory networks associated with FcγR signalling *in vivo*
3. Identify major FcγR-expressing cells within the GI tract and how FcγR expression changes with inflammation
4. Determine the effect of FcγR cross-linking on intestinal macrophage activation and its ability to induce Th17-polarising cytokines
5. Investigate the contribution of dysregulated macrophage FcγR signalling to the severity of intestinal inflammation in a murine model of DSS-induced colitis

1.10.2. Profiling Fcγ receptor expression and function on innate lymphoid cells

Hypotheses:

1. Intestinal ILC3s express FcγRs and IgG IC signalling induces ILC3 activation

The aims of this section of the thesis are to:

1. Determine the FcγR expression repertoire of intestinal ILC subsets
2. Investigate the effect of FcγR cross-linking on canonical FcγR signalling pathways and antigen internalisation
3. Determine the effect of FcγR signalling on the expression of cytokine and antigen presentation genes
4. Profile the effect of FcγR on global expression patterns by RNAseq

1.10.3. Innate lymphoid cells in the coordination of intestinal immunity

Hypotheses:

1. ILC cross-talk with co-resident intestinal immune cells is critical for intestinal immune defence

The aims of this section of the thesis are to:

1. Determine the effect of ILC depletion on defence against *C. rodentium* infection and DSS-induced colitis
2. Profile tissue-resident immune cell activation in the absence of ILCs
3. Identify key ILC-derived cytokines and chemokines that may regulate immune cell activation
4. Determine the mechanistic role of these mediators in immune cell activation
5. Determine the effectiveness of targeting ILC-derived inflammatory mediators in modulating similar effects *in vivo*

2. Methods

2.1. Mice

2.1.1. Overview

Mice were maintained in specific pathogen-free conditions at a Home Office-approved facility in the UK. All procedures were conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986.

2.1.2. Sourcing

All mouse lines used here are on a C57BL/6 background. C57BL/6 mice were obtained from Jackson Laboratories or from Charles River Laboratories (Margate, UK) and were used as controls. *Fcgr2b*-deficient mice on a C57BL/6 background were kindly provided by J. Ravetch (Rockefeller University) and S. Bolland (US National Institutes of Health, US National Institute of Allergy and Infectious Diseases (NIAID)). M-TG mice and non-transgenic (N-TG) controls were bred in-house. FcγRIIB overexpression was achieved using a construct in which FcγRIIB mRNA was placed under the control of the CD68 promoter [205]. RORγt-eGFP mice were kindly provided by D. Littman (New York University School of Medicine, USA). *Rag2*-deficient mice were kindly provided by Alex Betz (MRC Laboratory of Molecular Biology, UK).

2.1.3. Genotyping

Mice were identified and genotyped using ear punch biopsies. For genotyping, tissue was digested overnight at 55°C using DirectPCR (Tail) lysis reagent (Viagen) containing 0.3 mg/ml proteinase K (Sigma-Aldrich). Samples were then heat-inactivated at 86°C for 1 h. Digested samples were subjected to a touchdown polymerase chain reaction (PCR), as outlined in Table 2.1, using MyTaq reagent (Bioline) and the primers listed in Table 2.2 (Sigma-Aldrich). Samples were subsequently mixed with Orange G DNA loading buffer (BioVision) and run on a 1.5 % agarose gel (1.5 g agarose (BioGene) in 100 ml Tris/Borate/ ethylenediaminetetraacetic acid (EDTA) buffer (distilled H_2O containing 1.08 % wt/vol Tris, 0.55 % wt/vol boric acid, 2 mM EDTA) containing SYBR Safe (Thermo Fisher Scientific) alongside a lane containing HyperLadder 50bp (Bioline).

Table 2.1. Genotyping touchdown PCR protocol.

Step	Temperature (°C)	Time (min)	Notes
1	95	3	
2	95	0.5	Repeat steps 2-4 29 more times, decreasing from 60°C by 0.5°C per cycle
3	60	0.5	
4	72	0.5	
5	95	0.5	Repeat steps 5-7 29 more times
6	45	0.5	
7	72	0.5	
8	72	7	
9	12	Infinity	

Table 2.2. Genotyping primers.

Strain	Primer	Sequence (5' to 3')
FcγRIIB-KO	Forward 1	CTCGTGCTTTACGGTATCGCC
	Forward 2	AAACTCGACCCCCCGTGGATC
	Reverse	TTGACTGTGGCCTTAAACGTG
M-TG	Forward	TTCTCGCCTCTGTGCCTGACA
	Reverse	CAGCCCTCTCTTGGAAAGGAGC

2.2. *In vivo* procedures

2.2.1. Induction of experimental DSS-induced colitis

For all *in vivo* colitis experiments, 6 to 12-week old sex-matched mice were used. For all M-TG and *Rag2* knock-out colitis experiments, sex-matched littermate controls were used. FcγRIIB-deficient mice were matched with littermate controls (*Fcgr2b*$^{+/+}$ and *Fcgr2b*$^{-/-}$ mice from two *Fcgr2b*$^{+/-}$ breeders) or C57BL/6 mice obtained from Jackson Laboratories and maintained in-house. In cases where non-littermate controls were used, mice were co-housed for at least 3 weeks prior to the induction of DSS to minimise cage effects.

Acute colitis was induced by administration of 2 % wt/vol 36,000-50,000 molecular weight DSS (MP Biomedicals) as drinking water for 7 days. Colitis severity was monitored daily through changes in body weight, stool consistency, and intestinal haemorrhage, with a scoring system for clinical severity devised as outlined in Table 2.3. Moderate severity limits were imposed, with 20 % weight loss or two moribund characteristics judged to be the severity threshold.

Table 2.3. Clinical scoring system.

Weight loss (%)		Stool consistency		Intestinal haemorrhage	
Observation	**Score**	**Observation**	**Score**	**Observation**	**Score**
<0	1	Normal	0	No bleeding	0
0-5	2	Loose	1	Positive hemoccult	1
5-10	3	Watery diarrhoea	2	Slight visible bleeding	2
10-15	4			Substantial bleeding	3
15-20	5			Gross bleeding	4
>20	6				

Chronic colitis was induced by subsequent re-administration of 2 % wt/vol DSS for a further 6-7 days after a recovery period of two weeks following the first course of DSS treatment.

At experimental endpoints, the small and large intestines, spleen, MLNs and blood were harvested from all mice and colitis severity further assessed through morphological changes in key organs. The spleen, MLNs, and colon were weighed to monitor enlargement, and colon length measured from cecum to rectum to determine shortening. The tissues were then processed for histology, RNA extraction, or flow cytometric analysis.

2.2.2. *Citrobacter rodentium* infection (with Simon Clare)

Rag2-KO mice were orally infected with 200 µl of *C. rodentium* ICC180 (10^9 colony forming units (CFUs)). At day 7, mice were sacrificed and colon processed for flow cytometry and RNA analysis. Caecum, liver, and spleen were homogenised and bacterial burden assessed by serial dilution and plating onto agar plates. CFUs were determined after overnight incubation at 37°C.

2.2.3. *In vivo* antibody dosing

For *in vivo* antibody administration, all dosing was via intraperitoneal injection in a final volume of 200 µl PBS. All antibodies, except anti-IL-1R1 IgG, were administered on day 0 and day 3 of a 7-day protocol in the quantities listed in Table 2.4. Anti-IL-1R1 IgG was administered on day 0 and day 7 of a 15-day DSS protocol.

Table 2.4. *In vivo* dosing monoclonal antibodies

Antibody	Clone	Company	Dose (mg)
InVivoMAb anti-CD90.2 IgG	30H12	BioXCell	0.25
InVivoMAb anti-KHL IgG	LTF-2	2BScientific	0.25
Anti-*Escherichia coli* IgG	-	Abcam	0.5
InVivoMAb anti-IL-1R1 (CD121a) IgG	JAMA-147	BioXCell	1
InVivoMAb anti-GM-CSF IgG	MP1-22E9	BioXCell	0.125

2.2.4. Bleeds

2.2.4.1. Terminal cardiac bleeds

Exsanguination was carried out by direct puncture of the left atrium using a 26-gauge needle (BD Microlance) and 1 ml syringe (Terumo) following CO_2-induced euthanasia.

2.2.4.2. Tail-vein bleeds

Mice were placed in a 40°C heat-box for 10 min to induce dilation of the tail vein. Mice were restrained and exsanguination of the tail vein carried out using a U-100 insulin syringe (Braun).

2.2.5. Euthanasia

At experimental endpoints, or when exceeding severity limits, murine euthanasia was carried out by a two-step process, in accordance with local regulations. This was a combination of rising CO_2 concentration, cervical dislocation, exsanguination, or the onset of rigor mortis.

2.3. Tissue sample preparation

2.3.1. Spleen

Splenic single cell suspensions were obtained by forcing the tissues through a 70 µm cell strainer with ice-cold PBS. The resulting suspension was incubated with red blood cell (RBC) lysis buffer (distilled H_2O containing 0.83 % wt/vol ammonium chloride, 0.1 % wt/vol sodium hydrogen carbonate, 100 µM EDTA) for 1 min before washing twice in ice-cold PBS.

2.3.2. Lymph nodes

MLNs were cleaned of any residual fat and digested for 20 min at room temperature (PBS, 1 % fetal calf serum (FCS), 100 µg/ml DNase I (Sigma-Aldrich), 1 mg/ml Collagenase A (Sigma-Aldrich)). The remaining tissue was then passed through a 70 µm strainer and washed twice with ice-cold PBS.

2.3.3. Large and small intestine

Intestines were isolated and the associated fat and luminal contents gently removed. The tissues were subsequently opened longitudinally, cut into small pieces and washed by vortexing in ice-cold PBS with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Epithelial cells and intraepithelial lymphocytes were removed by shaking the tissues twice in stripping buffer (RMPI-1640 medium, 2 % FCS, 10 mM HEPES, 1 mM dithiothreitol (DTT; Sigma-Aldrich), and 5 mM EDTA) at 37˚C for 20 min. The underlying tissues were then digested for 30 min at 37˚C with an enzyme cocktail (0.42 mg/ml Liberase (Sigma-Aldrich) or

1 mg/ml Collagenase A (Sigma-Aldrich), with 60 ug/ml DNase I (Sigma-Aldrich)) in RPMI-1640 medium (Sigma-Aldrich), vortexed, and forced through a 70 µm cell strainer to break up any remaining tissue. The remaining intestinal suspensions were resuspended in RPMI-1640 containing 40 % Percoll (Sigma-Aldrich) and overlaid onto a solution of 80 % Percoll. Samples were then centrifuged at 600 *g* for 20 min at 10°C. Lamina propria mononuclear cells (LPMCs) were harvested at the 40 %/80 % interface and washed twice in ice-cold PBS containing 3 % FCS.

2.3.4. Blood

For serum collection, blood was collected in BD Microtainer serum tubes (BD) and incubated for 15 min at room temperature. Samples were then centrifuged at full speed for 10 min in a micro-centrifuge (Thermo Fisher Scientific). Serum was collected as the fluid top layer.

For analysis of leukocytes in blood, blood was collected in EDTA-coated Microvette tubes (Sarstedt) and subjected to RBC lysis prior to processing for flow cytometry, as outlined below.

2.3.5. Bone marrow

Bone marrow was flushed from the femur, tibia and humerus of mice using ice-cold sterile PBS and the subsequent cell suspension treated with RBC lysis buffer. Treated cells were then washed in ice-cold sterile PBS before proceeding to downstream applications.

2.3.6. Human ileum

The collection of human samples occurs under strict guidance from local surgical teams and National Health Service Blood and Transplant (NHSBT). Ethical approval was granted by the local ethics committee (National Research Ethics Service East of England committee – reference number: 15/EE/0152). Risk assessments for the handling of human tissue have been fulfilled according to local guidelines.

Human LPMC isolation was carried out in a similar way to murine LPMC isolation. Ileal samples were opened longitudinally and cleaned of luminal contents. The mucosa was manually dissociated from the muscular layers, cut into small pieces and washed by vortexing in ice-cold PBS with 10 mM HEPES. Epithelial cells and intraepithelial lymphocytes were removed by incubation in stripping buffer (RMPI-1640 medium, 2% FCS, 10 mM HEPES, 1 mM DTT, and 5 mM EDTA) at 37˚C for 1 h. The underlying tissues were then digested for 1 h at 37˚C with an enzyme cocktail (0.42 mg/ml Liberase or 1 mg/ml Collagenase A, with 60 ug/ml DNase I) in RPMI-1640 medium, vortexed, and mechanically dissociated using a Gentle-MACS machine (Miltenyi Biotech, Bisley, UK). Dissociated tissue was then passed through a 70 µm cell strainer and centrifuged for 10 min at 1300 rpm. Intestinal tissue suspensions were resuspended in RPMI-1640 containing 40 % Percoll (Sigma-Aldrich) and overlaid onto a

solution of 80 % Percoll. Samples were then centrifuged at 600 *g* for 20 min at 10°C. LPMCs were harvested at the 40 %/80 % interface and washed twice in ice-cold PBS containing 3 % FCS before proceeding to downstream applications.

2.4. Flow cytometry

2.4.1. Extracellular staining

For extracellular staining, cells were blocked with 0.5 % heat-inactivated normal mouse serum (NMS) for 20 min, followed by extracellular staining for 1 h at 4°C with a combination of the antibodies listed in Table 2.5 (murine) and Table 2.6 (human). All antibodies were used at a 1:200 dilution.

Table 2.5. Murine extracellular staining antibodies

Antibody	Clone	Company
B220	RA3-6B2	eBioscience
CD3e	145-2C11	eBioscience
CD4	GK1.5	eBioscience
CD11b	M1/70	eBioscience
CD11c	N418	eBioscience
CD19	1D3	eBioscience
CD25	PC61.5	eBioscience
CD45.2	104	eBioscience
CD80	16-10A1	eBioscience
CD86	GL1	eBioscience
CD90.2	30-H12	eBioscience
CD117/cKit	2B8	eBioscience
CD127	A7R34	eBioscience
CX3CR1	SA011F11	Biolegend
F4/80	BM8	eBioscience
FcγRI	X54-5/7	Biolegend
FcγRIIB	AT130-2	eBioscience
FcγRII/III	2.4G2	BD biosciences
FcγRIII	275003	R&D systems
FcγRIV	Gift from Mark Cragg (Southampton)	
GL7	GL7	eBioscience
IgA	-	SouthernBiotech
IgD	11-26	eBioscience
IgG	-	SouthernBiotech
IgM	eB121-15F9	eBioscience
KLRG1	2F1	eBioscience
Ly6C	HK1.4	eBioscience
Ly6C/G	RB6-8C5	eBioscience
MHC class II	M5/114.15.2	eBioscience
NK1.1	PK136	eBioscience
NKp46	29A1.4	BD biosciences
PD-L1	MIH5	eBioscience
TCR beta	H57-597	eBioscience
TCR gamma/delta	GL3	Biolegend

Table 2.6. Human extracellular staining antibodies

Antibody	Clone	Company
CD14	61D3	eBioscience
CX3CR1	2A9-1	eBioscience
FcγRI	10.1	eBioscience
FcγRIIA/B	CD32	eBioscience
FcγRIIIA/B	eBioCB16	eBioscience
IgA1/2	G20-359	BD biosciences
IgG	HP6017	Biolegend

2.4.2. Viability staining

Viability staining was then performed with LIVE/DEAD Fixable Aqua Dead Cell Stain kit (Thermo Fisher Scientific) for 20 minutes at room temperature. Following staining, samples were run fresh in PBS, stored in fixative (PBS containing 0.02 % wt/vol sodium azide, 2 % wt/vol glucose, 0.01 % wt/vol formaldehyde), or subjected to intracellular staining (see below).

2.4.3. Intracellular staining

For intracellular TF staining, cell fixation and permeabilisation was carried out using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience). For intracellular cytokine staining, cells were incubated in RPMI-1640 medium containing 10 % FCS and 1X penicillin-streptomycin (both Sigma-Aldrich, Gillingham, UK), hereafter referred to as complete RPMI (cRPMI), for 3 h with 1X Brefeldin A (eBioscience) solution, prior to fixation and permeabilisation using the Cytofix/Cytoperm kit (eBioscience). For intracellular phospho-staining, cells were fixed using BD Cytofix fixation buffer (BD biosciences) and permeabilised using BD Phosflow Perm buffer III (BD biosciences). All kits were used as per the manufacturer's instructions. Staining was then carried out for 1 h at room temperature using a combination of the antibodies listed in Table 2.7. All antibodies were used at a 1:100 dilution.

Table 2.7. Murine intracellular staining antibodies

Antibody	Clone	Company
FoxP3	FJK-16s	eBioscience
GM-CSF	MP1-22E9	eBioscience
IL-17A	TC11-18H10.1	Biolegend
IL-22	IL22JOP	eBioscience
Ki67	SolA15	eBioscience
Phospho-ERK	20A	BD biosciences
Phospho-SYK	moch1ct	eBioscience
Pro-IL-1β	NJTEN3	eBioscience
RORγt	AFKJS-9	eBioscience
T-bet	4B10	eBioscience

For cell counting, 123count eBeads (eBioscience) were added to single cell suspensions prior to antibody staining. Flow cytometry data collection was performed on a Fortessa cytometer (BD biosciences).

2.4.4. Flow sorting strategies

For ILC3 flow-sorting, small and large intestinal ILC3s were identified as lineage negative (CD3e, CD5, CD19, B220, Gr-1, CD11b, CD11c) $CD45^+$ $ROR\gamma t^+$ cells using RORγt-eGFP reporter mice. In non-reporter mice, intestinal ILC3s were identified as $lineage^-$ $CD45^+$ $KLRG1^-$ $c\text{-}Kit^+$ cells. Murine intestinal macrophages were sorted as $CD11b^+$ $F4/80^+$ $CX3CR1^+$ cells in uninflamed mice, while inflammatory monocytes and mature macrophages were identified as $CD11b^+$ $CX3CR1^+$ $Ly6C^{hi}$ $MHC\text{-}II^-$ and $CD11b^+$ $CX3CR1^+$ $Ly6C^{lo}$ $MHC\text{-}II^{hi}$ cells, respectively, in mice following DSS administration. Human ileal macrophages were flow-sorted as SSC^{int} $CX3CR1^+$ $CD14^+$ cells.

Cell sorting was performed on FACS Aria (BD biosciences), iCyt Synergy (Sony Biotechnology Inc.), and MoFlo (Beckman Coulter) cell sorters. Data were analysed using FlowJo software (Tree Star Inc.).

2.4.5. Microbial flow cytometry

Flow cytometry of antibody-bound commensal bacteria was carried out as previously described [477]. For murine samples, faecal contents were homogenised and briefly centrifuged at 1,000 rpm to remove large aggregates, and the resulting supernatant was washed twice in sterile PBS by centrifugation for 1 min at 8,000 rpm. Bacterial pellets were resuspended in sterile PBS containing 1:50 dilution of mouse serum or PBS alone in 96-well v-bottom plates and incubated for 20 min at 4°C. For human faecal samples, no serum opsonisation step was included. Bacteria were washed by centrifugation in sterile PBS at 3,000 rpm for 10 min in a bench-top Sorvall centrifuge. Bacterial pellets were then resuspended in PBS containing anti-mouse/human IgA or anti-mouse/human IgG antibodies and stained for 20 min at 4°C. Cells were washed and resuspended in PBS or fixative containing 1:10,000 SYBR Green (Invitrogen) and analysed by flow cytometry. For murine studies, faecal and serum samples were paired from the same mouse. Only SYBR Green-high events were analysed.

Human UC and control faecal samples were previously collected and supplied by Miles Parkes (Department of Gastroenterology, Addenbrooke's hospital, UK). Sample collection was carried out with the appropriate ethical approval. Human disease severity was determined previously by Parkes and colleagues using the Walmsley clinical activity index (CAI). Patient information, including medication regimen, is listed in Table 2.8.

Table 2.8. Patient information for samples used for microbial flow cytometry

Patient	Visit number	Date of sample collection	Walmsley CAI	Type of flare	CRP (mg/L)	Medication
1	1	19/04/2015	12	9 days; intermediate	72	-
2	2	22/04/2015	1	Still active	<4	Prednisolone (15 mg) Salofalk (3 g)
3	2	04/05/2015	6	Active but improving	-	Inpatient Co-trimoxazole (960 mg) Cyclosporine (225 mg) Hydrocortisone i.v. (50 mg) Ciprofloxacin tablet (500 mg) metronidazole tablet (400 mg)
4	1	13/10/2015	13	3-4 months	<4	Inpatient Methylprednisolone (40 mg) Hydrocortisone (SOLU-CORTEF) 100 mg
5	5	06/10/2015	5	Active but improving	None	Adcal Asacol (2.4 g) Prednisolone (35 mg)
6	13	09/09/2015	5	Active but improving	<4	asaTHIOprine tablet (100 mg) Methylprednisolone (40 mg)

2.4.6. Mitochondrial staining

Mitochondrial staining was carried out using MitoTracker Green FM (MG; Invitrogen). Adherent macrophages were incubated for 30 min at 37°C in RPMI containing 1X MitoTracker Green FM. Media was subsequently removed and the cells resuspended in PBS for analysis by flow cytometry.

2.5. *In vitro* stimulation assays

2.5.1. Macrophage generation

BMDMs were generated by incubation of bone marrow cells in cRPMI, and supplemented with 100 ng/ml murine recombinant M-CSF (Peprotech). M-CSF-supplemented cRPMI was replaced on day 3 and BMDMs were harvested on day 5. BMDMs were plated at least 4 h

prior to stimulation. In certain experiments, BMDMs were primed with 20 ng/ml murine recombinant GM-CSF (Peprotech) for 16 h prior to downstream stimulations.

2.5.2. Immune complex generation

Model ICs were generated in two ways. Insoluble ovalbumin IC (O-IC) was generated by the opsonisation of 40 µg/ml endotoxin-free ovalbumin (OVA; Invitrogen) with 1.2-2 mg/ml polyclonal rabbit anti-OVA IgG antibody (Sigma-Aldrich) in PBS at 37°C for 1h. Soluble IC was generated by the opsonisation of 60 µg/ml murine IgG (Abcam) with 600 µg/ml goat anti-mouse IgG Fab (Thermo Fisher Scientific) in PBS at 37°C for 1 h.

2.5.3. IC stimulation

Immune cells were stimulated with IC for 4 h for RNA analysis, including RNAseq and quantitative PCR (qPCR), 6 h for intracellular cytokine staining, or 16 h for enzyme-linked immunosorbent assays (ELISAs) at 37°C in a 5 % CO_2 incubator.

Flow-sorted intestinal ILC3s and MNPs were plated in cRPMI at a density of $5x10^3$-$1x10^5$ cells per well in 96-well plates prior to stimulation. LPMC suspensions and BMDMs were plated in cRPMI at a density of $5x10^5$-$1x10^6$ cells per well prior to stimulation. Wells were additionally supplemented in various experiments with 50 ng/ml IL-23 (Peprotech), 20 ng/ml IL-7 (Peprotech), 20 ng/ml IL-1β (Peprotech), 100 ng/ml IFNγ (Peprotech), 100 ng/ml TNF (Peprotech), and 200 ng/ml TL1A (R&D Systems). Cells were then washed extensively, and analysed by qPCR, ELISA, and flow cytometry.

2.5.4. Phagocytosis assay

Flow-sorted ILC3s were incubated at 37°C for 16 h with 10 µg/ml OVA-647 (Thermo Fisher Scientific) or O-IC-647 generated as above. Phagocytosis was assayed by flow cytometry.

2.6. Immunofluorescence

Murine spleens, MLNs, PPs, and intestinal tissues were fixed with 1 % paraformaldehyde (Electron Microscopy Services) in PBS for 16 h, washed with PBS, and equilibrated in 30 % wt/vol sucrose for a further 16 h. Tissues were then frozen at -80°C in Optimal Cutting Temperature embedding medium (Thermo Fisher Scientific).

Cryostat sections were cut at a thickness of 20-30 µm, air dried for 1 h, then rehydrated for 10 min in PBS and blocked with a 0.1 M Tris solution containing 1 % vol/vol NMS, 1 % wt/vol BSA, and 0.1 % wt/vol Triton X-100 for 1 h at room temperature. Sections were stained overnight at 4°C with a combination of the antibodies listed in Table 2.5 in blocking buffer at a 1/100

dilution. Additionally, actin was stained in certain experiments using Phalloidin dyes (Thermo Fisher Scientific) at a 1/100 dilution.

Confocal imaging was carried out on a Leica SP8 confocal microscope. Images were analysed using Imaris software (Bitplane).

2.7. RNA analysis

2.7.1. Extraction and reverse transcription

RNA extraction was carried out using commercially available kits as per the manufacturer's instructions. QIAGEN RNeasy micro kits were used for cell numbers below 5 x 10^5. PureLink mini kits were used for cell numbers over 5x10^5. For whole tissue RNA extraction, tissue pieces were first disrupted using a Precellys 24 Homogenizer (Bertin Instruments), before extraction using the PureLink mini kit. RNA concentration and purity was determined using a NanoDrop spectrophotometer (Thermo Scientific) prior to cDNA synthesis using a High-Capacity RNA-to-cDNA kit (Applied Biosystems).

2.7.2. Quantitative polymerase chain reaction

All qPCR was carried out in triplicate with Taqman reagents and pre-designed TaqMan Gene Expression Assay primers and probes (Thermo Fisher Scientific), as listed in Tables 2.9 (murine) and 2.10. (human). qPCR was performed on the Viia 7 PCR machine (Life Technologies, Paisley, UK). Gene expression was normalised to *Gapdh* or *Hprt* using the $2^{-\Delta Ct}$ method [615]. The $2^{-\Delta\Delta Ct}$ method was used for normalisation between experimental conditions and genotypes.

Table 2.9. Murine qPCR primers

Target gene	Assay ID
Ahr	Mm00478932_m1
Ccl2	Mm00441242_m1
Ccl8	Mm01297183_m1
Csf2	Mm01290062_m1
Cxcl1	Mm04207460_m1
Cxcl2	Mm00436450_m1
Fcer1g	Mm02343757_m1
Fcgr1	Mm00438874_m1
Fcgr2b	Mm00438875_m1
Fcgr3	Mm00438882_m1
Fcgr4	Mm00519988_m1
Gapdh	Mm99999915_g1
Hprt	Mm03024075_m1
Il1b	Mm00434228_m1
Il6	Mm00446190_m1
Il10	Mm01288386_m1
Il17a	Mm00439618_m1
Il22	Mm01226722_g1
Il23a	Mm00518984_m1
Reg3a	Mm01181787_m1
Reg3b	Mm00440616_g1
Reg3g	Mm00441127_m1
Tnf	Mm00443258_m1
Tnfsf15	Mm00770031_m1

Table 2.10. Human qPCR primers

Target gene	Assay ID
CXCL8	Hs00174103_m1
FCER1G	Hs00175408_m1
FCGR1B	Hs02341825_m1
FCGR2A	Hs01013401_g1
FCGR2B	Hs01634996_s1
FCGR3A	Hs02388314_m1
FCGR3B	Hs04334165_m1
GAPDH	Hs02786624_g1
HPRT1	Hs02800695_m1
IL1B	Hs01555410_m1

2.8. Soluble protein assays

2.8.1. Cytokine and chemokine analysis

Quantification of cytokines and chemokines in culture supernatants was carried out using commercially available R&D systems Duoset ELISA kits, as per the manufacturer's instructions.

2.8.2. Serum anti-commensal IgG quantification

Measurement of anti-commensal IgG titres in murine sera was carried out as previously described [587]. Colonic faecal contents were processed as described in *Microbial flow cytometry*. Bacteria were homogenised using BugBuster (Novagen), centrifuged at 20,000 *g* for 10 min, and the supernatant recovered for a crude commensal bacterial antigen preparation. Protein concentration was determined using the Pierce BCA protein assay kit (Thermo Fisher Scientific). Subsequently, a 96-well Nunc ELISA plate (Thermo Fisher Scientific) was coated with 5 µg/ml commensal antigen preparation overnight at 4°C, washed extensively, and murine sera incubated in doubling dilutions for 4 h at room temperature. For small volumes of sera, samples were incubated at a 1:150 dilution. In the case of serum anti-flagellin IgG detection, 96-well NUNC plates were coated overnight with 200 ng/ml flagellin. Commensal antigen-specific IgG was detected using a goat anti-mouse IgG-horseradish peroxidase (HRP) conjugated antibody (Thermo Fisher Scientific, 1:10000 dilution), and tetramethylbenzidine (TMB) peroxidase substrate (BD biosciences). After 15-20 min, the reaction was quenched with 1 M Na_2SO_4 and the optical densities measured at 450 nm using a plate spectrophotometer.

2.8.3. Luminal IgG quantification

Murine colonic luminal contents were extruded using bicarbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$) at point of death. 96-well Nunc ELISA plates (Thermo Fisher Scientific) were coated with primary goat anti-murine IgG or goat anti-murine IgA antibodies (catalogue numbers 1037-01 and 1040-01, respectively; SouthernBiotech) for 16 h at 4°C. Plates were extensively washed and incubated with luminal suspensions in serial dilutions for 4 h at room temperature. Bound IgG and IgA were detected using secondary goat anti-murine IgG and goat anti-murine IgA antibodies conjugated to HRP (catalogue numbers 1037-05 and 1040-05, respectively; SouthernBiotech) and TMB peroxidase substrate (BD biosciences). Ig concentration was determined using a standard curve of murine IgG (I5381-5mg; Sigma-Aldrich) or murine IgA (14-4762-81; Thermo Fisher Scientific). Ig levels were normalised to total luminal protein content, as determined using the Pierce BCA protein assay kit (Thermo Fisher Scientific).

2.9. RNAseq and microarray analyses (with John Ferdinand)

2.9.1. RNAseq

2.9.1.1. Cell sorting and RNA extraction

Following O-IC stimulation, approximately $2x10^4$ RORγt$^+$ ILC3s and $1x10^5$ CX3CR1$^+$ macrophages per condition were transferred into 750 µl RLT plus buffer (Qiagen). Sample

eppendorfs were immediately vortexed, snap frozen on dry ice and stored at -80°C. RNA was extracted from cell lysates using the RNeasy plus micro kit (Qiagen) as per the manufacturer's instructions. Optimal DNA depletion columns were used to remove contaminating genomic DNA. Purified RNA was eluted in nuclease free water (Ambion) and stored at -80°C.

2.9.1.2. RNA quality control

Quality and concentration of the purified RNA was assessed using an RNA pico chip (Applied Biosystems) using a Bioanalyzer 2000 (Applied Biosystems) as per the manufacturer's instructions. For all RNAseq experiments, samples had an RNA integrity number greater than 8, indicating minimal degradation of the RNA.

2.9.1.3. Library preparation and QC

For the preparation of libraries, SMARTer stranded total RNA-Seq mammalian pico input kit (Takara) was used as per the manufacturer's instructions. To produce the libraries, 1.5-3.55 ng of total RNA was used and libraries were amplified for 14 cycles of PCR. Library size was assessed using 1 µl of undiluted final libraries with a High Sensitivity DNA chip (Applied Biosystems) using a Bioanalyzer 2000 (Applied Biosystems) as per the manufacturer's instructions. Library concentration was quantified by PCR using 1/10000 dilution of the library in nuclease free water (Ambion) with ROX low KAPPA library quantification kit (KAPPA Biosystems). Libraries were pooled at an equimolar concentration with up to 12 libraries per pool.

2.9.1.4. Sequencing

Sequencing of the libraries was carried out using a Hiseq 2500 (Illumina) on a 2x100bp sequencing run with 1 pool per flow cell lane. Sequencing was carried out at Genewiz (NJ, USA).

2.9.1.5. Analysis

Pooled libraries were de-multiplexed by Genewiz using Casava (Illumina) before transfer of the data to the University of Cambridge. Fastq files were trimmed of the first 3 nucleotides of the R1 strand and also to remove contaminating adaptor sequences and poor-quality bases (bases with a phred 33 score of <30) using trimgalore! (Babraham bioinformatics) and quality of the resulting files was assessed using FastQC (Babraham bioinformatics). Fastq files were aligned to the mm10 genome (Downloaded from https://genome-euro.ucsc.edu/cgi-bin/hgGateway?db=mm10&redirect=manual&source=genome.ucsc.edu on 18th January 2016) using hisat2. All analysis was carried out using R version 3.2.4. Reads were counted and assigned to genes using the Featurecount function from the RSubread package.

Differential expression analysis was carried out using DESeq2 using a linear model with an appropriate design matrix following the default workflow. Resulting figures were plotted using ggplot2 and heatmap.2 from the gplots package. Gene-set enrichment analysis (GSEA) was performed for RNAseq data by first assigning a rank metric to each gene using the following formula:

$$\text{Rank metric} = 1(P \text{ value} + 1\text{x}10^{-300})*(|\text{LFC}|/\text{LFC})$$

GSEA was then run using GSEA 2.1 using the pre-ranked option with the classic setting against gene sets from the Molecular Signature Database (MSigDB; http://software.broadinstitute.org/gsea/msigdb). FcγR gene sets are listed in Table S1, Appendix.

STRING analysis was run using the online GUI using the multiple protein entry and the high confidence (score > 0.7) setting.

2.9.2. Microarray

Publicly available microarray datasets were downloaded from GEO (https://www.ncbi.nlm.nih.gov/geo/) along with appropriate chip annotation data. All analyses were carried out using R. All datasets were downloaded as raw intensity matrices. Data was normalised using RMA. Probes were reduced to one probe per gene by selecting the probe with the greatest variance across the samples using the gene filter package. Differential expression was varied out using limma with an appropriate design matrix. Graphics were produced using the same programmes as for the RNAseq analysis above.

Listed in Table 2.11 are the publicly-available transcriptomic datasets that were used throughout Chapters 3 to 6.

Table 2.11. GEO transcriptomics datasets

Accession number	Dataset	Additional information
GSE9452	Endoscopic pinch biopsies from the descending colon of ulcerative colitis patients and control subjects.	8 UC samples with macroscopic signs of inflammation. 13 UC samples without macroscopic signs of inflammation. 5 control subjects.
GSE38713	Endoscopic pinch biopsies from UC patients with histologically active UC and inactive UC, as well as non-inflammatory controls. Sample ages ranged from 18-65.	Active UC defined as Mayo sub score ≥ 2 and Matts ≥ 3. Inactive UC defined as Mayo sub score = 0 and Matts ≤ 2 and a remission state for a minimum for 5 months prior to and 6 months post-collection. Uninvolved mucosa from patients with active UC defined as normal endoscopic and histological appearance and absence of evidence of previous disease. 15 involved active UC; 7 non-involved active UC; 8 inactive UC; 13 healthy controls.
GSE12251	Endoscopic pinch biopsies from 22 clinically active UC patients prior to infliximab treatment. Patients subsequently stratified on successful response.	Response to infliximab defined as endoscopic and histological healing at week 8. 12 responders and 10 non-responders.
GSE42768	Total RNA from snap-frozen colon from mice with acute or chronic relapsing colonic inflammation induced using several cycles of exposure to DSS in drinking water.	Data analysed from H_2O-treated healthy controls (n = 5) and mice after 1 or 2 cycles of DSS with an intervening period of 2 weeks (n = 5 in each case).
GSE37448	Gene-expression microarray datasets generated as part of the Immunological Genome Project.	
GSE71253	RNA extracted from human CD14 monocytes cultured in RPMI and 10 % FCS supplemented with either GM-CSF or M-CSF for 7 days.	
GSE42101	RNA from flow-sorted colonic lamina propria mononuclear phagocyte subsets under steady state and inflammatory conditions.	

2.9.3. Hierarchical clustering

R and the built-in stats package were used for all calculations. Plots were generated using the package dendextend. For clustering analysis, the RMA normalised intensity for the genes of interest (all cytokines (Table S1, Appendix) plus *FCGR2A*) were selected and a distance matrix calculated using Euclidean distance using the function “dist”. The samples were subsequently hierarchically clustered using the function “hclust” using the complete method. To define

clusters, the tree was cut into k clusters at a given height based on visual interpretation of the dendrogram (typically k=4/5). The cluster containing *FCGR2A* was examined further.

2.10. Statistics

Statistical analysis was performed using GraphPad Prism software for most experiments. For *in vivo* colitis experiments, comparison between experimental groups was performed using a nonparametric Mann-Whitney test. A parametric Student's *t* test was used for *in vitro* stimulation experiments, unless paired samples were used, where a ratio paired *t* test was used. For correlations of RNA expression levels, linear regression analysis was used. For bioinformatics analyses, *P* values were calculated using the standard DESeq 2 method with multiple correction using BH. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

3. IgG and FcγR signalling in intestinal inflammation

3.1. Introduction and hypotheses

IBD is a chronic, relapsing condition that is increasing in prevalence worldwide and causes considerable morbidity. There are two main subtypes, CD and UC, that differ in their clinical and pathological presentations, but are both thought to be driven by a predisposition to aberrant immune responses to commensal microbes [234]. IBD is associated with the production of numerous pro-inflammatory cytokines, including TNFα, and GWA studies have identified polymorphisms in the Th17 pathway as a major risk factor in both UC and CD [154], [262]. Blockade of IL-12p40 (a common subunit in IL-12 and IL-23) imparts a degree of protection in patients with infliximab-refractory disease, demonstrating a pathogenic role for this pathway in IBD, while elevated IL-1β levels are associated with severe infliximab-refractory IBD [334], [364], [365].

One of the strongest non-HLA associations with UC is a SNP in *FCGR2A*, a human activating FcγR found on a variety of immune cells, including macrophages, DCs, and neutrophils [64], [154], [156]. This SNP (rs1801274) encodes a histidine-to-arginine (H/R) substitution within the IgG-binding domain of FcγRIIA (FcγRIIA-R131) that results in reduced receptor affinity for IgG, and confers significant protection against the onset of UC. This supports the hypothesis that IgG plays a pathogenic role in IBD. Recent studies have demonstrated that anti-commensal IgG generation is a normal function of the adult immune system, with roles in maternally-derived immunity in the foetus, immune cell development, and the protection from systemic pathogen challenge [477], [479], [616]. However, seminal work by Per Brandtzaeg, and later Blumberg and colleagues, has demonstrated that anti-commensal IgG antibodies are significantly increased in IBD and can drive intestinal inflammation in murine models of colitis [427], [482]. Indeed, IgG-producing gut plasma cells are found within the inflamed mucosa of patients with both UC and CD [430]. Furthermore, the nature of IBD-associated IgG is different from healthy individuals, including alterations in the IgG glycome [127]. However, cellular mechanistic insights into the pathogenic role of IgG in colitis are lacking.

A link has been established between FcγR signaling *in vitro* and *in vivo* with the induction of IL-1β and subsequent induction of Th17 immune responses, raising the possibility that FcγR signaling may contribute to a major IBD susceptibility pathway [74], [75], [183]. We therefore hypothesized that FcγR signaling may contribute to intestinal inflammation via activation of mucosal Th17 immunity.

3.2. IgG within the gastrointestinal tract

To first address the concept of IgG as a local mediator of inflammation in the GI tract, analysis of transcriptomic datasets was carried out. Biopsies taken from the descending colon of UC

patients demonstrated an enrichment of *IGHG1* transcripts, as well as those for *IGHM* and *IGHD,* relative to healthy controls (HCs) (Fig. 3.1A). In contrast, *IGHA1* transcript levels were unchanged, resulting in an increase in the *IGHG1*/*IGHA1* ratio following the onset of inflammation (Fig. 3.1B). IgG1 is predominantly induced in response to protein antigens, exhibits high FcγRIIA binding relative to other IgG subclasses, and an ability to activate complement. Therefore, IBD is associated with the emergence of inflammatory IgG subclasses.

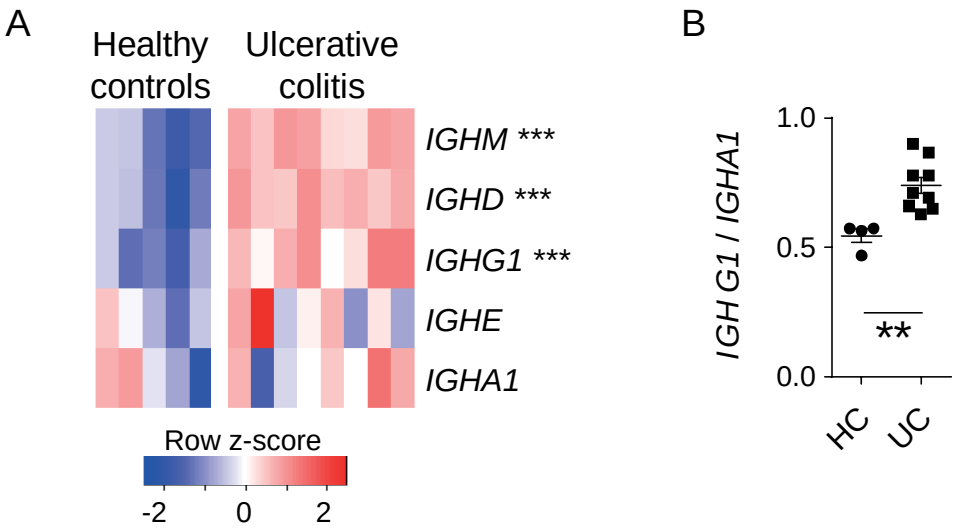

Figure 3.1. Transcriptomics analysis of *IGH* gene transcripts in human ulcerative colitis. (A) Heatmap showing transcriptomics analysis of human immunoglobulin heavy chain gene transcripts in colonic biopsies from UC patients and HCs. Data was derived from GEO dataset GSE9452. (B) The ratio of *IGHG1* to *IGHA* gene transcripts in UC patients and HCs; values derived from A. *P* values were calculated using limma with multiple correction using BH. *** $P < 0.001$.

In order to determine whether this increase in local *IGHG1* transcript levels reflects an increase in local anti-commensal IgG, flow cytometry was used to detect binding of antibodies directly to luminal bacteria (Fig. 3.2). Bacteria were identified as SYBR green^{hi} events, as previously described [477] (Fig. 3.2A). As expected, IgA1/2-bound bacteria were prevalent in healthy individuals, with little IgG binding, consistent with the role of IgA in homeostasis. In contrast, patients with UC exhibited a marked increase in IgG-bound bacteria within the lumen, demonstrating the induction of a potent anti-commensal IgG response in these patients (Fig. 3.2A). Interestingly, there was little change in bacteria-bound IgA1/2 levels in UC patients versus HHCs. Furthermore, the extent of IgG-bound bacteria reflected disease severity: UC patients with a Walmsley CAI greater than 10 had the greatest levels of IgG-bound bacteria (Fig. 3.2B). In contrast, there was no significant correlation between IgA binding and disease activity.

IBD is characterised by the increase in serum anti-commensal IgG antibodies. To examine this in a murine model of intestinal inflammation, we administered mice with DSS *ad libitum*, a chemical colitogen that causes osmotic epithelial damage within the colon. Mice developed *de novo* anti-commensal (Fig. 3.3A) and anti-flagellin (Fig. 3.3B) IgG serum antibodies, the latter being a dominant IBD-associated antigen.

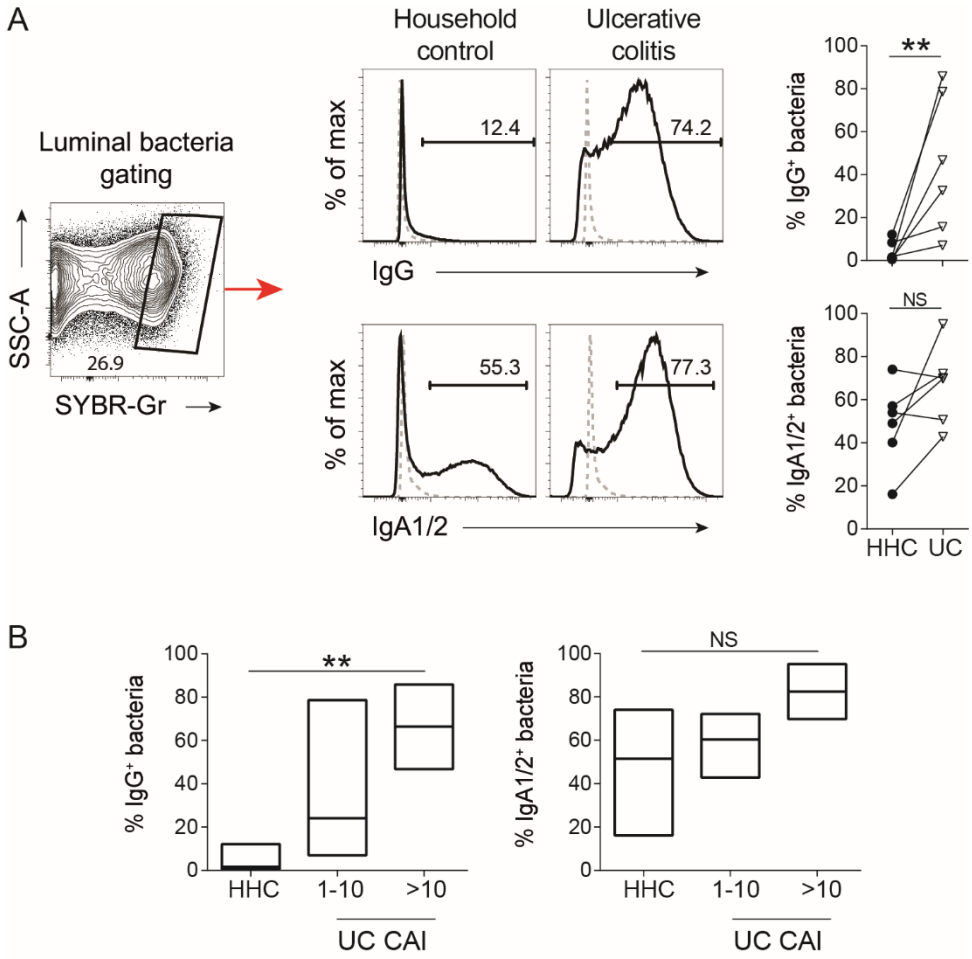

Figure 3.2. Enhanced anti-commensal IgG in human UC. (A) Microbial flow cytometry showing IgG and IgA1/2-bound SYBR green^{hi} bacteria in UC patients and healthy household controls (HHCs); analysed as pairs. (B) IgG and IgA1/2-bound bacteria quantification stratified based on the Walmsley CAI. HHC, n = 6; CAI 1-10, n = 4; CAI > 10, n = 2. Data was pooled from two independent experiments. P values were calculated using a ratio-paired t test (A) and the nonparametric Mann-Whitney test (B). ** P < 0.01.

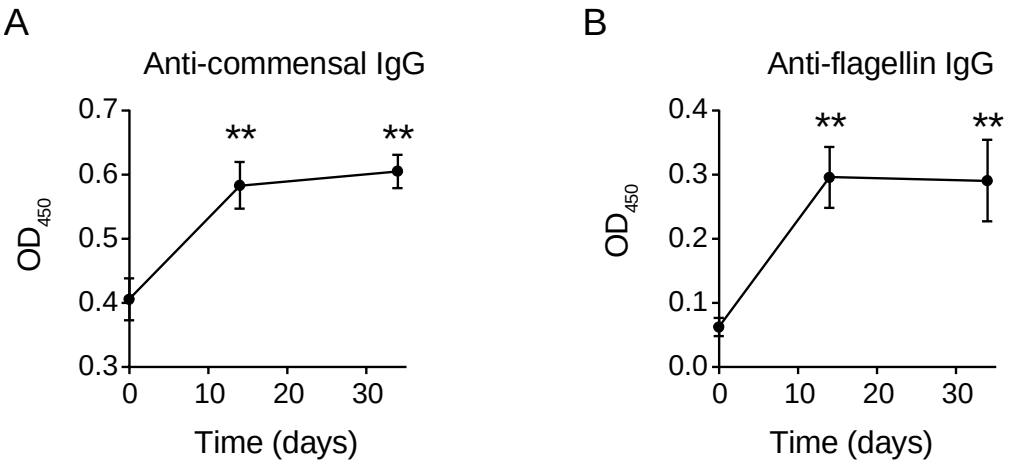

Figure 3.3. Serum anti-commensal IgG ELISA. (A and B) ELISAs showing de novo anti-commensal (A) and anti-flagellin (B) IgG generation following an acute course of DSS administration. *n* = 6. Data are representative of three independent experiments. *P* values were calculated using the nonparametric Mann-Whitney test. ** *P* < 0.01.

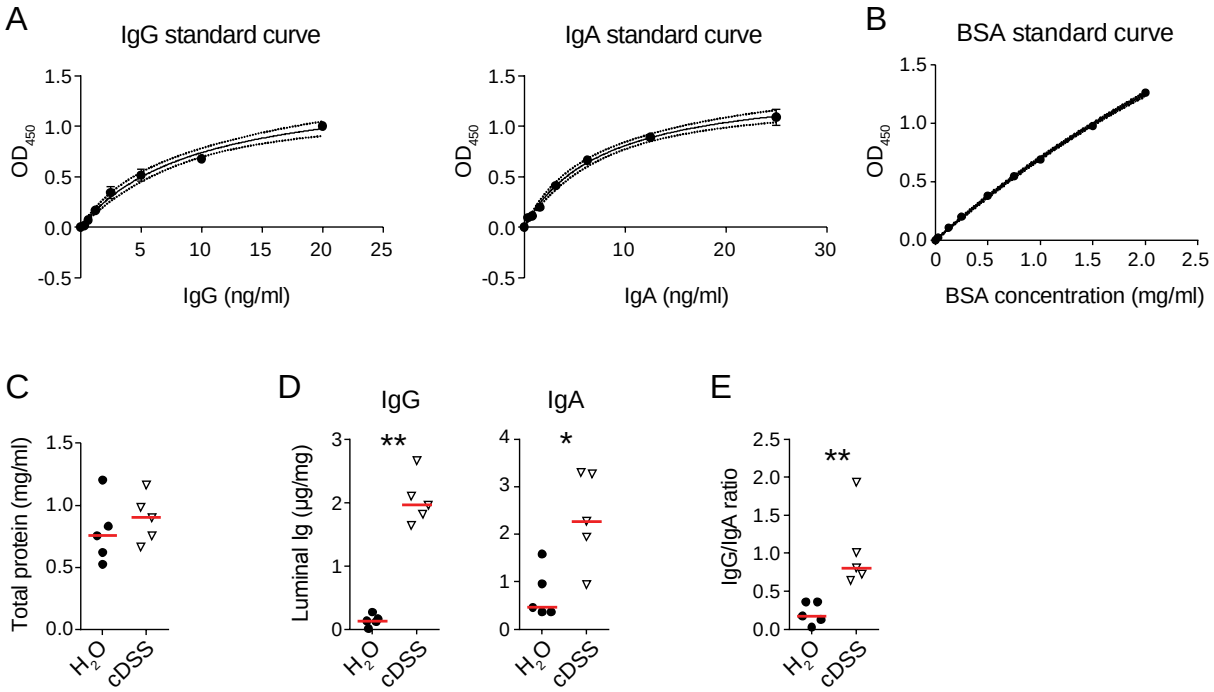

Figure 3.4. Enhanced luminal antibody levels following chronic DSS-induced colitis. (A) ELISA showing IgG and IgA standard curves for quantification of luminal protein levels. (B) BSA standard curve for quantification of total luminal protein content. (C) Luminal protein quantification in healthy mice treated with H_2O or following chronic DSS administration (cDSS). (D) Luminal IgG and IgA protein levels normalised to total protein in healthy mice and inflamed mice following cDSS. (E) IgG to IgA ratio based on ELISA readings. Data are representative of two independent experiments. *P* values were calculated using the nonparametric Mann-Whitney test. * *P* < 0.05; ** *P* < 0.01.

Luminal antibody levels were measured in gut washes from uninflamed and DSS-inflamed colons. Serial dilutions of luminal samples were compared to standard curves of recombinant murine IgG and IgA (Fig. 3.4A), and normalised to total protein content using a BSA standard curve (Fig. 3.4B). Total luminal protein content was largely similar between groups (Fig. 3.4C). However, while total IgG made up a small fraction of protein in uninflamed controls, this was significantly increased in the lumen of mice following a chronic course of DSS-induced colitis (Fig. 3.4D). Furthermore, a significant enrichment of IgA protein was also detected. Notably, however, the IgG/IgA ratio was increased during inflammation (Fig. 3.4E), mirroring the observation made in human IBD.

To determine whether this luminal increase in IgG reflected a directed anti-commensal response, a microbial flow cytometry assay was once again used. IgA binding was significant during homeostasis, with little change upon pre-incubation with paired serum (Fig. 3.5). IgG^+ bacteria were low under these conditions. However, incubation with paired serum resulted in a significant increase in IgG-bound commensal bacteria, in agreement with previous studies [477]. Following the onset of inflammation, IgG^+ bacteria were significantly increased within the lumen of mice, while no change was detected for IgA binding. Furthermore, pre-incubation with paired serum resulted in a more marked increase in IgG^+ bacteria compared to uninflamed mice, suggesting *de novo* anti-commensal IgG generation during colitis.

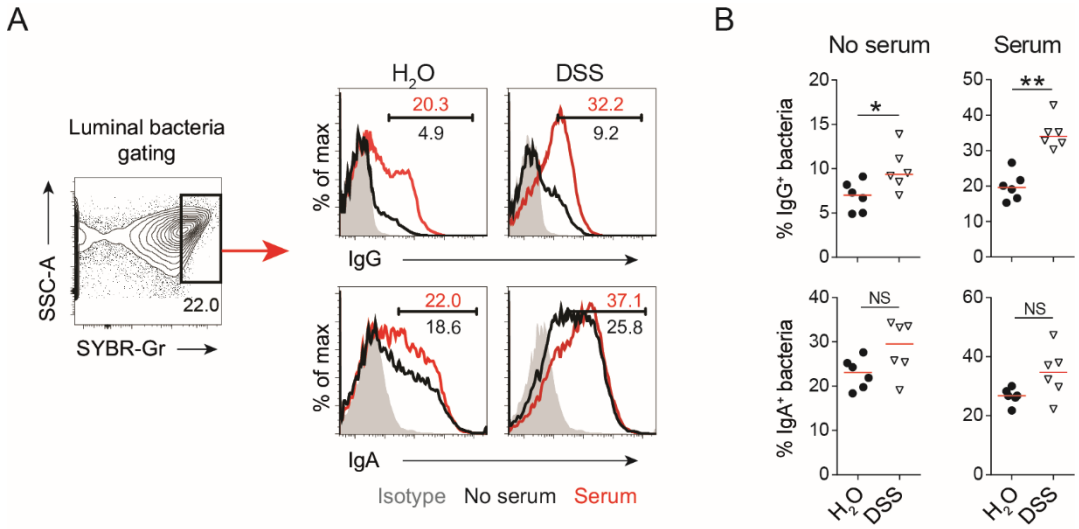

Figure 3.5. Enhanced IgG-bound commensal bacteria following chronic murine intestinal inflammation. (A) Microbial flow cytometry showing IgG and IgA-bound luminal SYBR green^{hi} commensal bacteria in uninflamed (H_2O) and inflamed murine colons following 7-day DSS administration. Grey = isotype control; black = non-serum-opsonised bacteria; red = serum-opsonised bacteria. (B) Quantification of microbial flow cytometry staining shown in A. Data are representative of two independent experiments. *P* values calculated using the nonparametric Mann-Whitney test. * $P < 0.05$; ** $P < 0.01$.

These results demonstrate that, while IgA is the dominant antibody isotype under homeostatic conditions in both humans and mice, there is a significant increase in *de novo*-generated anti-commensal IgG following the onset of intestinal inflammation. Furthermore, IgA levels do not significantly change, suggesting a skew towards the pro-inflammatory IgG isotypes during colitis.

3.3. Profiling intestinal B cell populations in intestinal inflammation

Given the increase in local IgG, we hypothesised that this was reflective of an increase in local IgG$^+$ B cells within the inflamed LP.

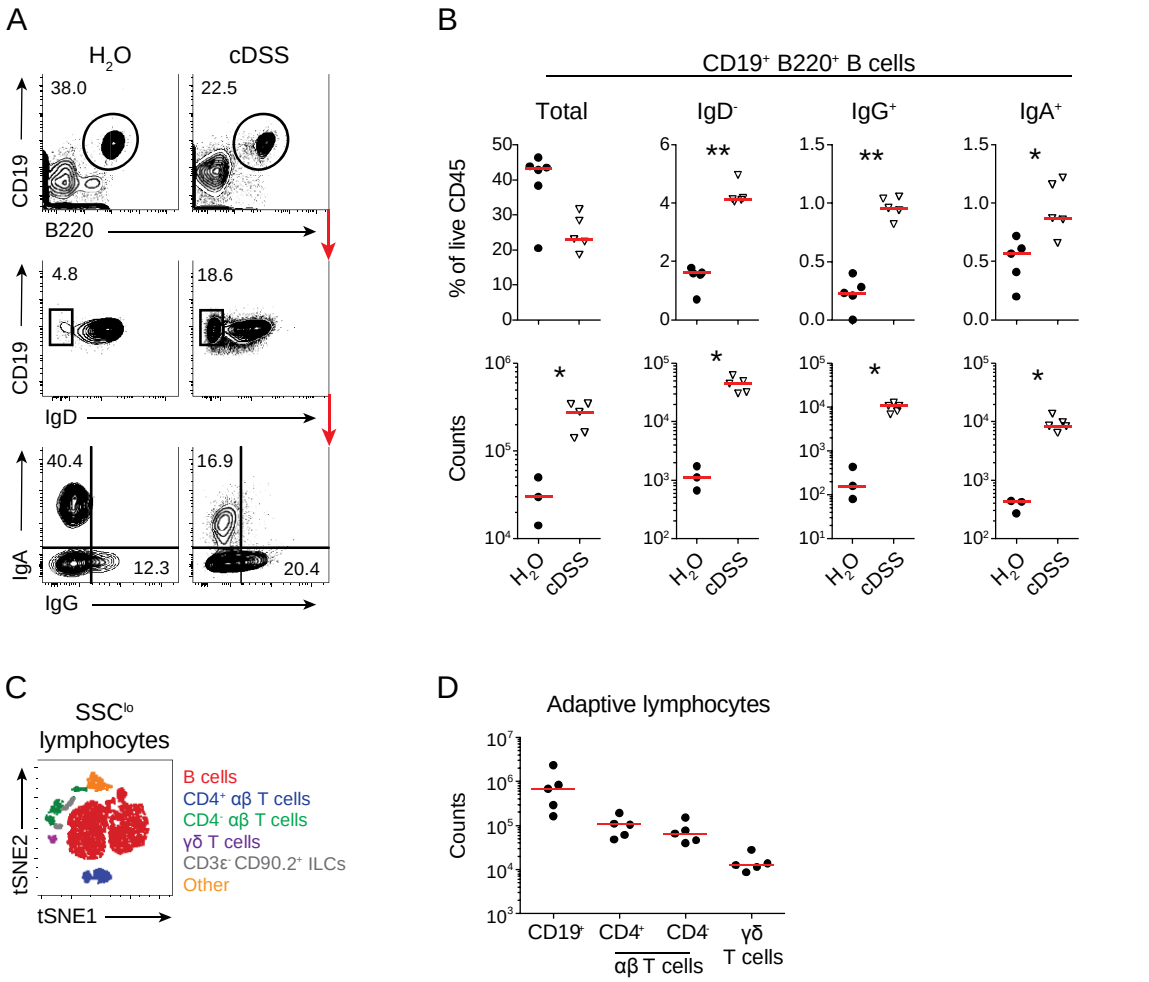

Figure 3.6. B cell infiltration characterises chronic intestinal inflammation. (A) Flow cytometry gating strategy for identification of class-switched B cells within the colonic lamina propria in uninflamed and cDSS-inflamed mice. (B) Quantification of total B cells (far left), IgD-negative B cells (middle-left), IgG-positive B cells (middle-right), and IgA-positive B cells (far right) in frequency and absolute number, as shown in A. (C) tSNE analysis of lymphocytes within the inflamed colonic lamina propria. (D) Quantification of adaptive lymphocytes 21 days following an acute course of DSS. Data are representative of two independent experiments. *P* values were calculated using the nonparametric Mann-Whitney test. * $P < 0.05$; ** $P < 0.01$.

Analysis of LP-resident B cell populations was carried out by flow cytometry. In contrast to uninflamed murine controls, there was a significant influx of $CD19^+$ $B220^+$ B cells into the LP following chronic DSS-induced colitis (Fig. 3.6A, B). Furthermore, a greater proportion of the infiltrating B cells were IgD^- (Fig. 3.6A, B). While IgA^+ B cells represented the major population of class-switched B cells in healthy controls, the frequency of IgG^+ B cells was significantly increased during inflammation, with equal numbers of IgG^+ and IgA^+ B cells. Furthermore, analysis of the frequency and absolute number of SSC^{lo} lymphocyte subsets in chronic DSS colitis demonstrated that B cells represent the major lymphocyte population within the inflamed mucosa (Fig. 3.6C, D). Confocal microscopy of colonic tissue sections from uninflamed or chronic DSS-inflamed mice demonstrated a pronounced influx of IgG^+ cells into the colon (Fig. 3.7). IgG^+ cells were localised predominantly within the inflamed submucosa, with scattered cells within the LP.

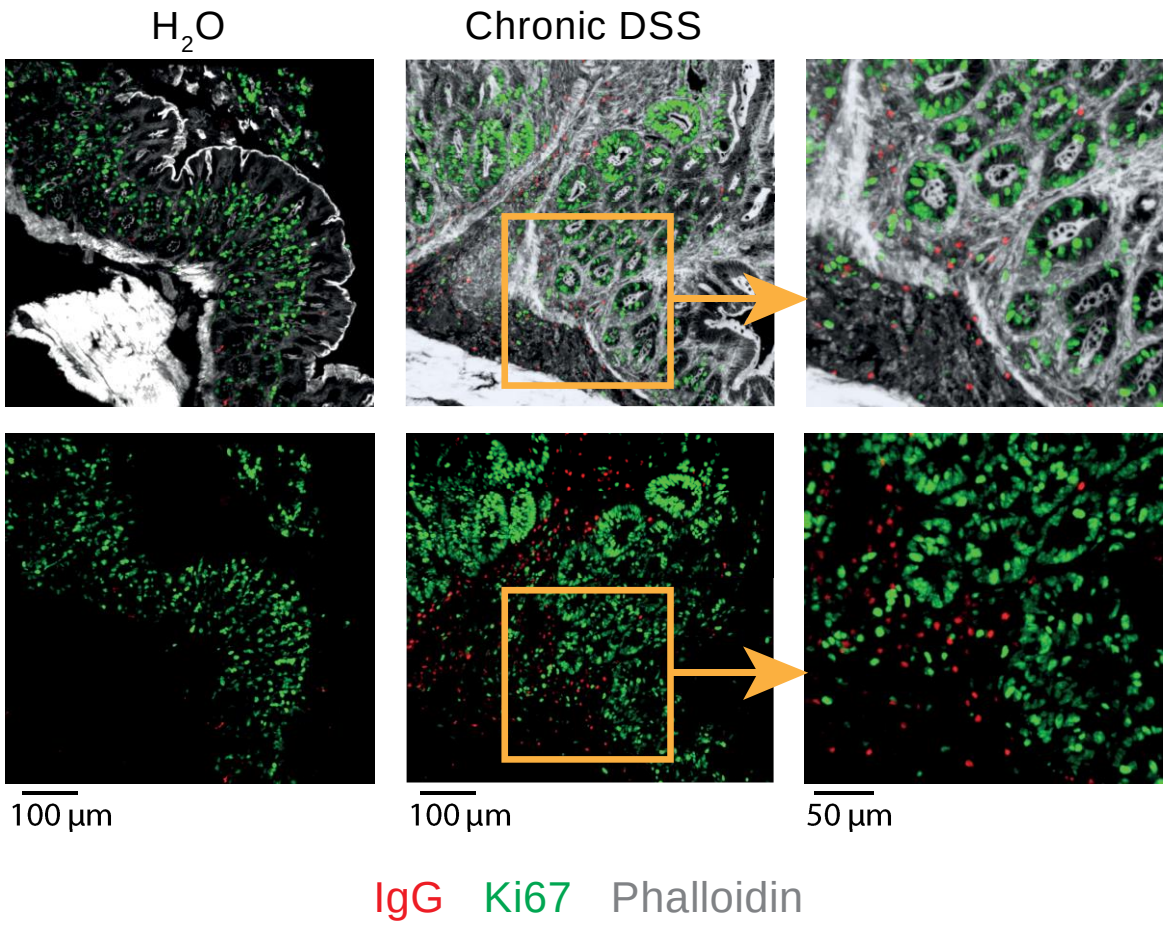

Figure 3.7. IgG^+ B cell identification within the inflamed colonic mucosa. Confocal microscopy showing IgG^+ B cells within the colonic mucosa following cDSS versus uninflamed controls (H_2O). White = Phalloidin; green = Ki67; red = IgG. Data are representative of two independent experiments.

These results demonstrate that colonic inflammation is characterised by the influx of class-switched and IgG$^+$ B cells within the mucosa. B cells represent the major lymphocyte population in this setting, and are located primarily within the submucosa and scattered throughout the LP.

3.4. Fcγ receptor signalling in intestinal inflammation

One of the key mechanisms by which IgG isotypes drive inflammation is through the engagement of Fcγ receptors. In particular, IC-mediated inflammation relies on the low-to-medium affinity FcγRs: activating FcγRIII and FcγRIV in mice, FcγRIIA, FcγRIIIA and FcγRIIIB in humans, and the inhibitory receptor FcγRIIB. First, we sought to determine whether FcγRs were enriched in the inflamed GI tract. Analysis of *FCGR* gene expression within colonic biopsies from patients with UC demonstrated enrichment of FcγR gene transcripts in accordance with the extent of inflammation (Fig. 3.8A). All activating FcγR transcripts showed significant enrichment, along with *FCER1G*, encoding the ITAM-bearing common γ-chain required for activating receptor signalling. Although significantly increased, the relatively smaller increased in *FCGR2B* expression resulted in a global increase in the A/I ratio in colitis (Fig. 3.8B). As such, global FcγR levels are skewed towards enhanced IgG responsiveness.

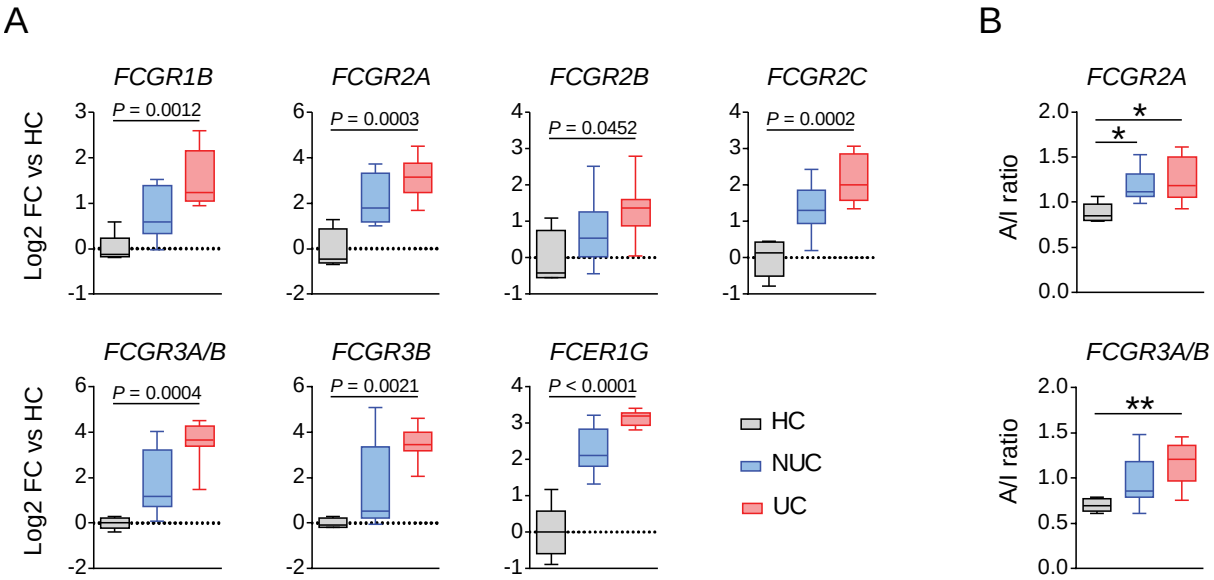

Figure 3.8. Fcγ receptor enrichment and increased global A/I ratios in UC. (A) Analysis of transcriptomics data of human Fcγ receptor genes in colonic biopsies from UC patients (red), non-inflamed UC patients (blue) or healthy controls (grey). Data were generated from the GEO dataset GSE9452. HC $n = 5$, NUC $n = 13$, UC $n = 8$. (B) Global A/I ratio of *FCGR2A* and *FCGR3A/B* gene transcripts for samples shown in A. *P* values were calculated using limma with multiple correction using BH (A) or the nonparametric Mann-Whitney test (B). * $P < 0.05$; ** $P < 0.01$.

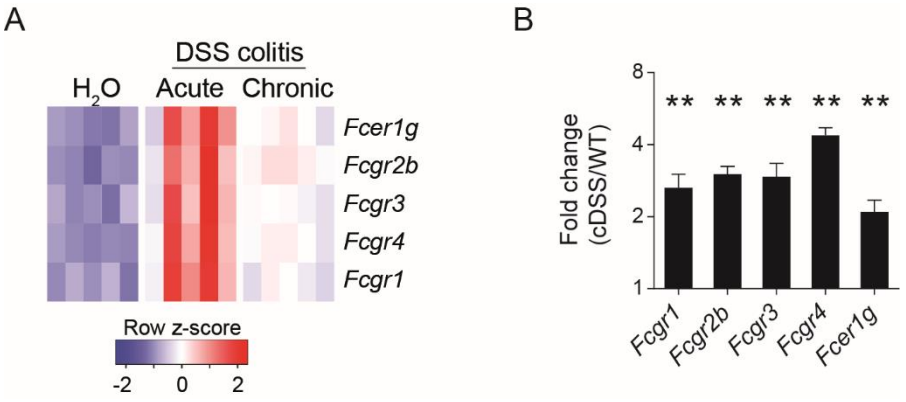

Figure 3.9. Colonic FcγR transcript enrichment in chronic murine DSS-induced colitis. (A) Heatmap showing transcriptomic analysis of murine FcγR gene transcripts in whole colonic tissue following acute and chronic DSS administration, versus healthy controls. Data were generated from GEO dataset GSE42768. (B) qPCR confirmation of murine FcγR gene transcripts in whole colonic tissue following cDSS, displayed as fold change compared to healthy control colonic tissue. $n = 6$ per group. qPCR data is representative of two independent experiments. *P* values were calculated using the nonparametric Mann-Whitney test. ** $P < 0.01$.

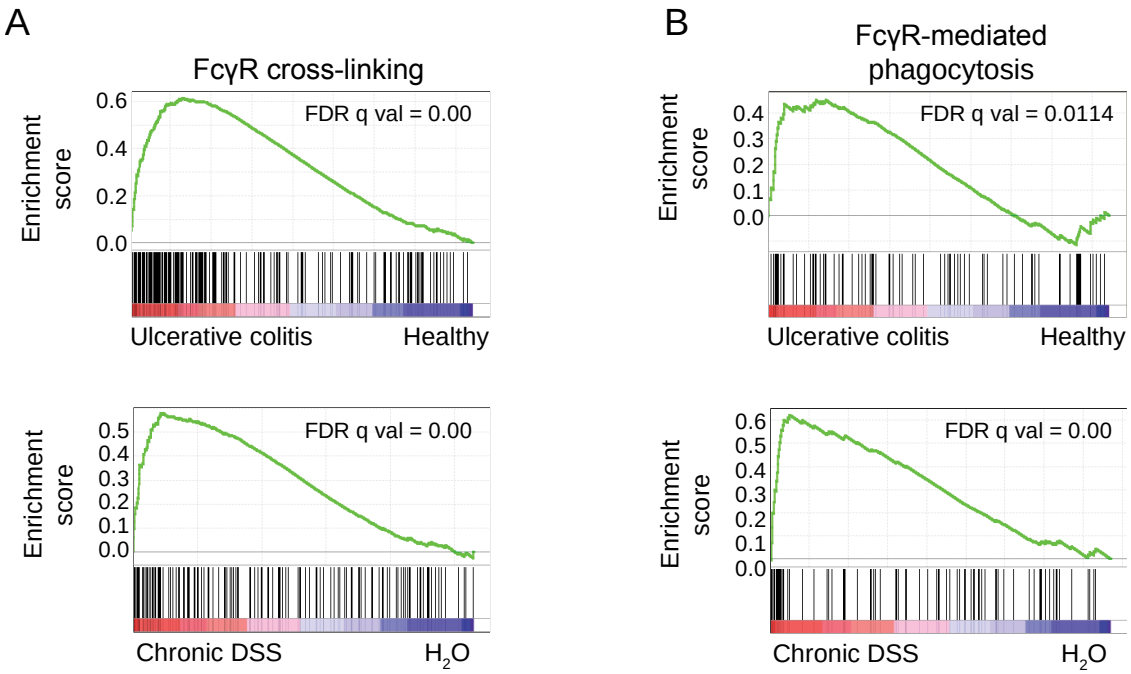

Figure 3.10. Enrichment of FcγR signalling pathways in chronic intestinal inflammation. (A) GSEA of FcγR crosslinking-associated genes in human UC colonic biopsies (top) and murine cDSS-inflamed whole colonic tissue (bottom) compared to healthy controls (GSE9452 = human IBD [UC = 8, HC = 5]; GSE42768 = murine DSS [H_2O = 5; xDSS = 5]). (B) GSEA of genes involved in FcγR-mediated phagocytosis in human UC colonic biopsies (top) and murine cDSS-inflamed whole colonic tissue (bottom) compared to healthy controls. Gene lists found in Table S1, Appendix.

A similar observation was made in murine DSS-induced colitis (Fig. 3.9). Transcriptomics analysis of whole colonic tissue following acute or chronic DSS administration (Fig. 3.9A), and subsequently confirmed by qPCR of whole colon tissue (Fig. 3.9B), demonstrated an increase in all *Fcgr* gene transcripts following the onset of inflammation.

GSEA was carried out to decipher whether FcγR-associated signalling pathways were similarly enriched (Fig. 3.10). Indeed, in both human UC and murine colitis, FcγR-associated signalling modules were significantly increased.

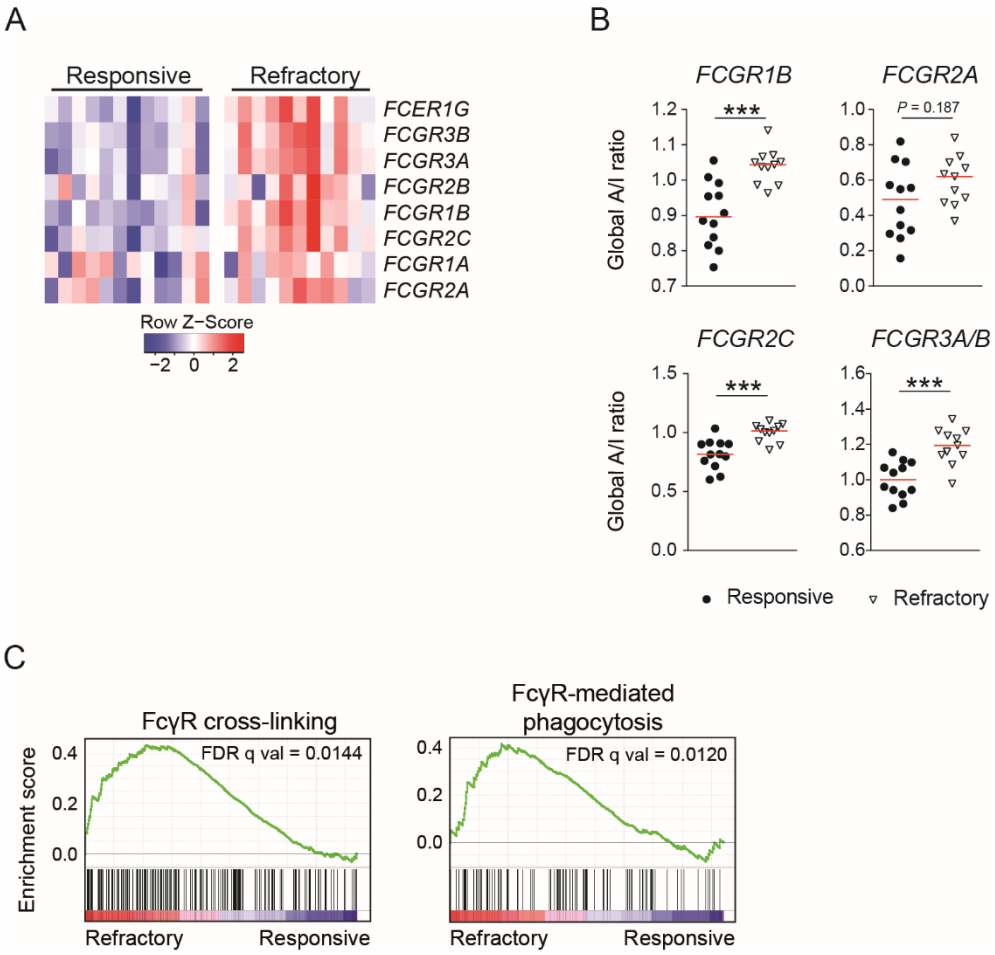

Figure 3.11. FcγR signalling is associated with infliximab-refractory UC. (A) Heatmap showing FcγR gene transcripts in colonic biopsies from patients responsive or refractory to infliximab therapy prior to the commencement of therapy. Data was derived from the GEO dataset GSE12251. (B) Global A/I ratios for activating receptors in colonic biopsies from patients shown in A. (C) GSEA of FcγR cross-linking-associated genes (left) and FcγR-mediated phagocytosis genes (right) in infliximab-responsive and refractory UC patients. Gene lists found in Table S1, Appendix. *P* values were calculated using the nonparametric Mann-Whitney test. *** $P < 0.001$.

Exacerbated FcγR signalling is associated with enhanced susceptibility to several inflammatory disorders, as demonstrated from human GWA studies and murine models [63], [64], [162], [206]. While infliximab administration is a cornerstone of IBD therapy, approximately 70 % of patients remain refractory to treatment in the long term. To determine whether FcγR signalling was associated with infliximab-refractory disease, the FcγR phenotype was analysed in these patients (Fig. 3.11). Compared to responsive individuals, infliximab-refractory patients demonstrated a local increase in FcγR transcripts (Fig. 3.11A), increased global A/I ratios (Fig. 3.11B), and the enrichment FcγR signalling pathways (Fig. 3.11C) in colonic biopsies prior to the beginning of treatment.

These results demonstrate that intestinal inflammation in humans and mice causes the local enrichment of FcγR signalling pathways. Furthermore, enhanced FcγR signalling is associated with increased disease severity, as demonstrated in infliximab-refractory UC patients, and may contribute to exacerbated inflammation in these individuals. These studies support the observation that commensal IgG binding correlates with disease activity.

3.5. Tissue-resident immune cell FcγR expression

Given the widespread expression of FcγRs and their regulation by the inflammatory milieu, the local enrichment of FcγR and signalling pathways could result from cellular infiltration and local modulation of FcγR expression within the inflamed GI tract.

To address this, we first sought to identify the major FcγR-expressing cell populations within the inflamed colon by flow cytometry (Fig. 3.12A). Within the $CD11b^{+}$ subset of cells, $Ly6C/G^{hi}$ $CX3CR1^{-}$ neutrophils, $CX3CR1^{+}$ $Ly6C^{lo\text{-}int}$ MNPs, including macrophages and monocytes, and $CX3CR1^{-}$ $MHC\text{-}II^{+}$ DCs represent major FcγR-expressing myeloid cells. Furthermore, SSC^{lo} $MHC\text{-}II^{+}$ B cells are known for their expression of FcγRIIB. A comparison of FcγR expression across these cell types demonstrated significant cell-to-cell variation (Fig. 3.12 B, C). As previously demonstrated, FcγRI expression is highly specific for murine intestinal $CX3CR1^{+}$ $Ly6C^{lo}$ $MHC\text{-}II^{hi}$ macrophages. Unexpectedly, intestinal macrophages expressed very high levels of inhibitory FcγRIIB, with less pronounced staining on DCs and B cells. Expression of the activating receptors FcγRIII and FcγRIV was also highest on macrophages, but also significantly expressed on neutrophils and DCs, and absent from B cells. Therefore, intestinal macrophages are uniquely characterised by their elevated levels of surface FcγRI and FcγRIIB.

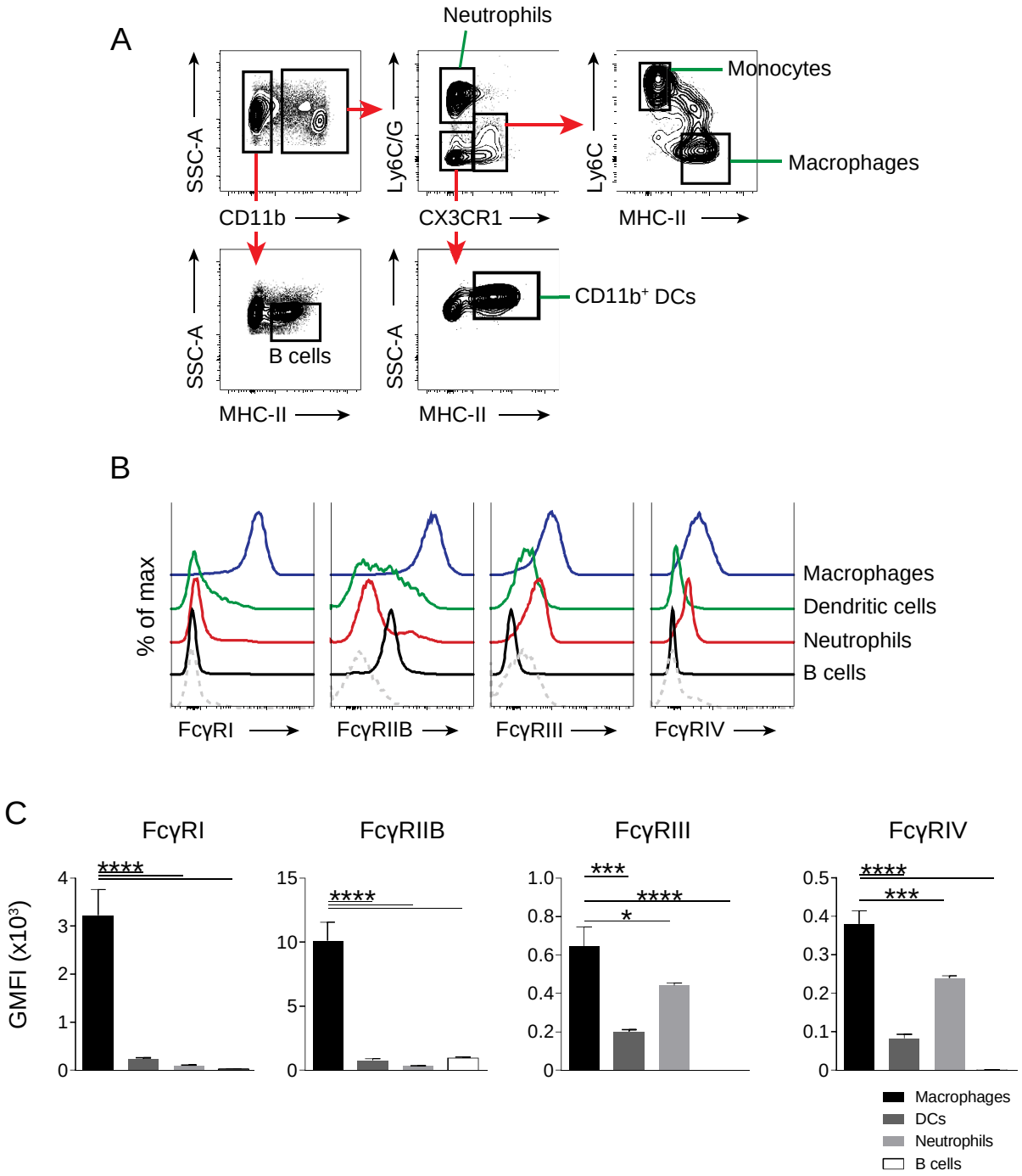

Figure 3.12. CX3CR1⁺ MHC-II^{hi} macrophages express the highest levels of FcγRs in the colonic LP. (A) Flow cytometry gating strategy to identify major FcγR-expressing cell types within the colonic LP. (B) Flow cytometry showing individual FcγR staining across the different cell types identified in A. (C) Quantification of FcγR staining intensity shown in B. *n* = 3 per group. Data are representative of two independent experiments. *P* values were calculated using parametric Student's t test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Intestinal tissue-resident macrophages are constantly replenished from circulating CCR2^{+} Ly6C^{hi} MHC-II^{-} monocytes. The maturation of monocytes into macrophages can be represented as a "waterfall" plot of CX3CR1^{+} MNPs by flow cytometry [68] (Fig. 3.13A): as monocytes mature, they downregulate Ly6C and upregulate MHC-II, resulting in Ly6C^{hi} MHC-II^{-} monocytes, Ly6C^{+} MHC-II^{+} monocytes/immature macrophages, and Ly6C^{lo} MHC-II^{hi} macrophages. As MNPs and neutrophils represent the major FcγR-expressing cells in the colon, we profiled their enrichment in DSS-induced colitis. DSS administration results in the influx of monocytes into the colonic LP and subsequent enrichment of all CX3CR1^{+} MNP subsets (Fig. 3.13A). Inflammation also results in local neutrophil recruitment (Fig. 3.13B). Therefore, influx of major FcγR-expressing immune cells contributes to FcγR enrichment in intestinal inflammation.

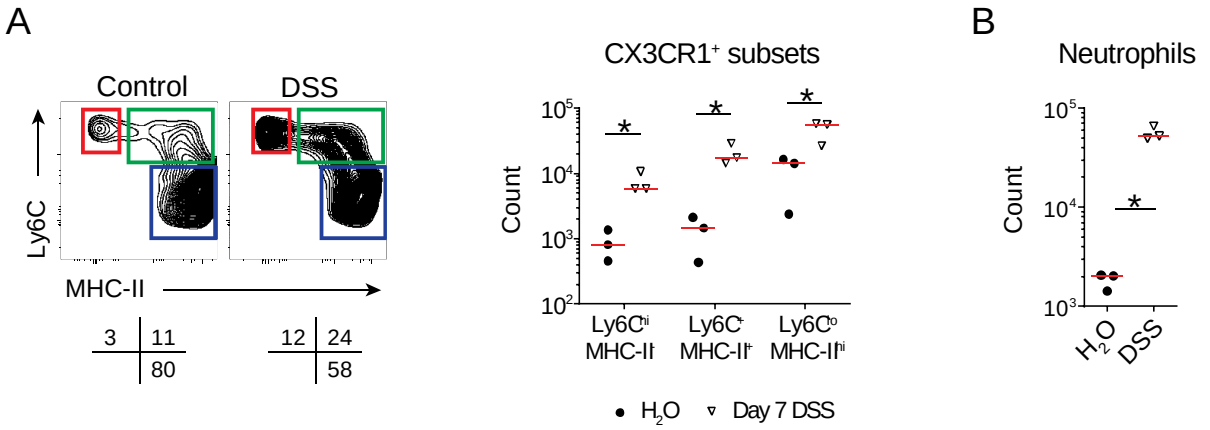

Figure 3.13. Cellular infiltration contributes to FcγR enrichment in intestinal inflammation. (A) Quantification of colonic CX3CR1^{+} MNP subsets by flow cytometry following DSS-induced colitis. (B) Quantification of neutrophilic infiltration into the colonic lamina propria following DSS administration. Data are representative of two independent experiments. *P* values were calculated using the nonparametric Mann-Whitney test. * $P < 0.05$.

Given the known effects of cytokines on FcγR expression by immune cells, we profiled CX3CR1^{+} MNP FcγR expression during homeostasis and DSS-induced inflammation (Fig. 3.14). FcγRI, FcγRIIB, and FcγRIV expression increased as cells matured from monocytes to macrophages, while FcγRIII expression remained relatively constant across all three MNP subsets (Fig. 3.14A). All activating FcγRs trended towards a significant increase in expression following the onset of intestinal inflammation (Fig. 3.14B, C). However, on macrophages, FcγRIIB expression decreased slightly with inflammation (Fig. 3.14 B, C), resulting in an increase in the A/I ratio of all activating FcγRs in this population (Fig. 3.14D). Therefore, tissue-resident and recruited macrophages are more primed to respond to local IgG following the induction of intestinal inflammation.

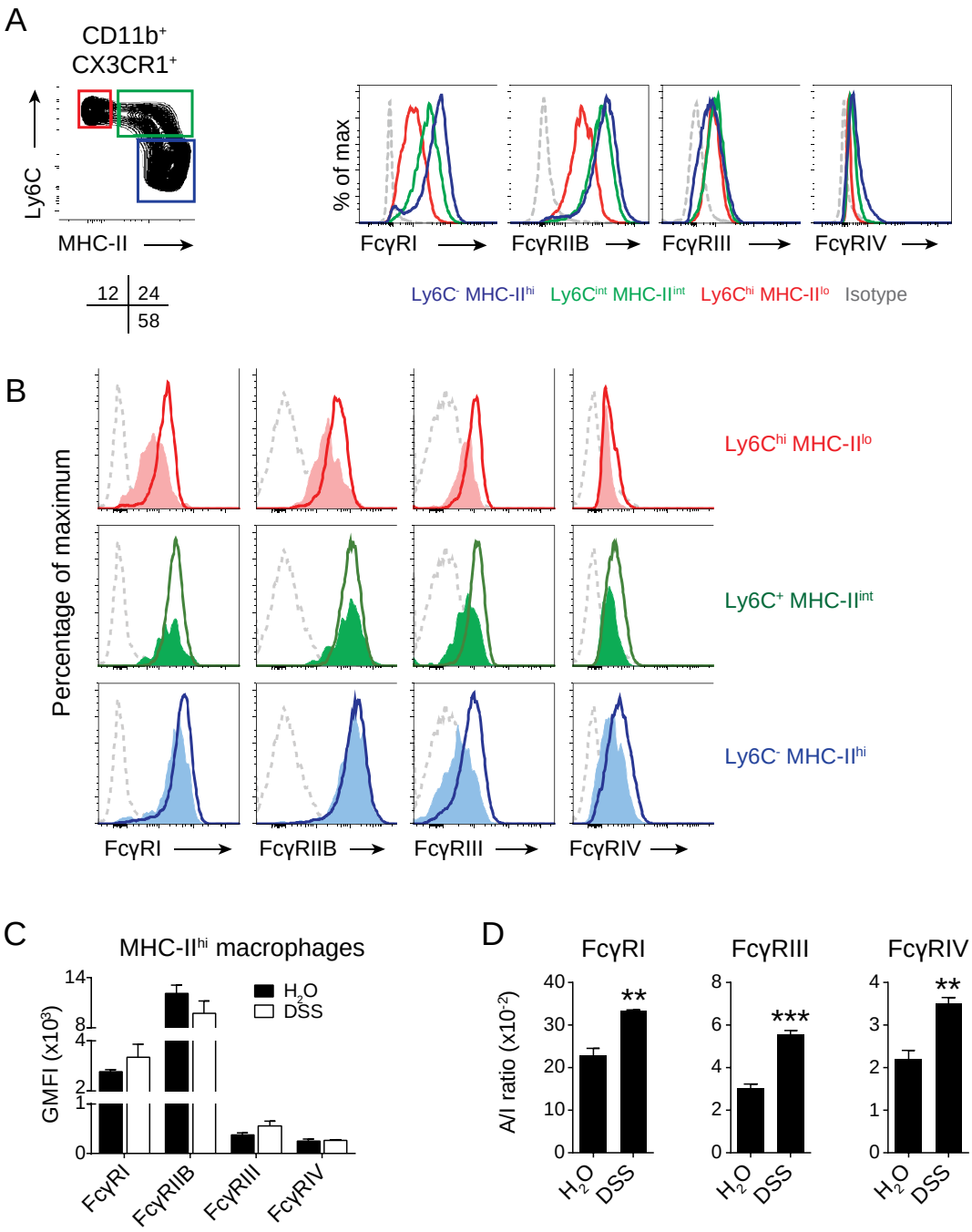

Figure 3.14. Altered macrophage A/I FcγR ratios in intestinal inflammation. (A) Flow cytometry staining of FcγRs on colonic CD11b$^+$ CX3CR1$^+$ MNP subsets. (B, C) Comparison of FcγR staining across CX3CR1$^+$ MNP subsets (B) and FcγR quantification of the MHC-II^{hi} macrophages (C) in healthy and DSS-inflamed colons. Red = monocytes (Ly6C^{hi} MHC-II^{lo}); green = MHC-II$^+$ monocytes (Ly6C$^+$ MHC-II$^+$); blue = macrophages (Ly6C^{lo} MHC-II^{hi}). Filled histrograms = H_2O-treated mice; open histograms = DSS-treated mice. *n* = 3 per group. (C) Quantification of macrophage A/I ratios based on flow cytometric staining intensity. Data are representative of two independent experiments. *P* values were calculated using the parametric Student's t test. ** $P < 0.01$; *** $P < 0.001$.

We sought to investigate how the FcγR repertoire of human intestinal macrophages compared to their murine counterparts. FcγR profiling of human ileal CX3CR1^{+} CD14^{+} macrophages by flow cytometry (Fig. 3.15A) and qPCR (Fig. 3.15B) demonstrated predominant expression of FcγRIIA, the major UC-associated FcγR, and FcγRIIB. Therefore, human and murine macrophages are characterised by elevated levels of both activating and inhibitory FcγRs, with the UC-associated FcγRIIA most highly expressed.

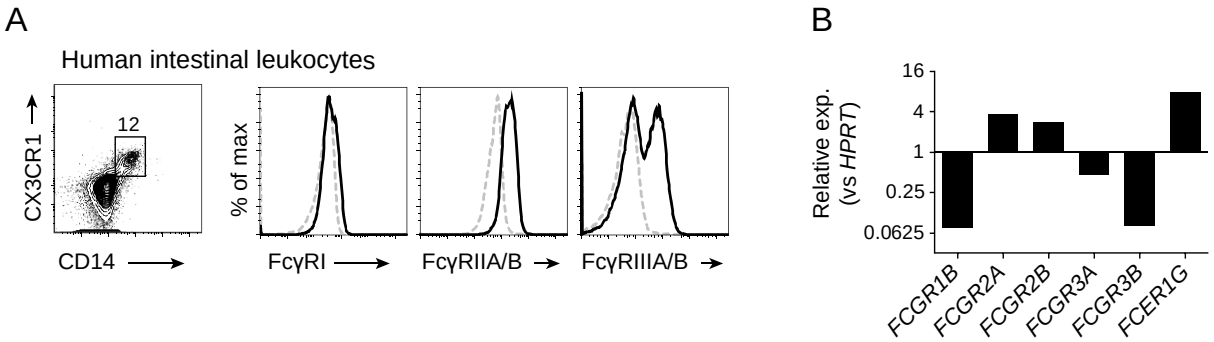

Figure 3.15. Fcγ receptor expression by human intestinal macrophages. (A) Flow cytometry FcγR staining of human ileal macrophages. (B) qPCR of FcγR genes in flow-sorted human ileal CX3CR1^{+} CD14^{+} macrophages. Data are representative of a sole experiment.

In summary, we have shown that intestinal CX3CR1^{+} Ly6C^{lo} MHC-II^{hi} macrophages represent the subset with the highest levels of FcγR expression within the murine colon, particularly FcγRI and FcγRIIB. Furthermore, local FcγR enrichment during inflammation results from the combined effects of leukocytic infiltration, as expected, and the local modulation of FcγR expression levels, with a skew towards an increased A/I ratio and elevated responsiveness to IgG following the onset of inflammation.

3.6. FcγR-associated inflammatory networks in intestinal inflammation

Aberrant Th17 immunity is strongly associated with intestinal inflammation, with the IL23R-Th17 signaling axis a major risk factor for the development of IBD [262]. We hypothesised that FcγR signalling, and in particular FcγRIIA, may contribute to aberrant Th17 immune responses within the inflamed mucosa through the direct regulation of macrophage-derived cytokines and chemokines.

To first address how FcγR signalling may contribute to IBD-associated inflammation, we sought to identify major cytokine and chemokine pathways enriched in colonic biopsies from UC patients versus healthy controls. Analysis of transcriptomic data demonstrated that several Th17-associated cytokines were significantly enriched in UC mucosa, with *IL1B* being the most upregulated cytokine with IBD (Fig. 3.16A). Other Th17-associated genes enriched in UC biopsies included *IL23A* and *IL17A*. Chemokine gene analysis demonstrated a nucleus of granulocyte-recruiting chemokines enriched in patients with UC, including *CXCL1*, *CXCL2*,

and *CXCL3*, and *CXCL8*, the prototypical neutrophil-recruiting chemokine (Fig. 3.16B). Therefore, UC is associated with aberrant Th17 immunity, in particular exacerbated IL-1β production, and the expression of neutrophil-recruiting chemokines.

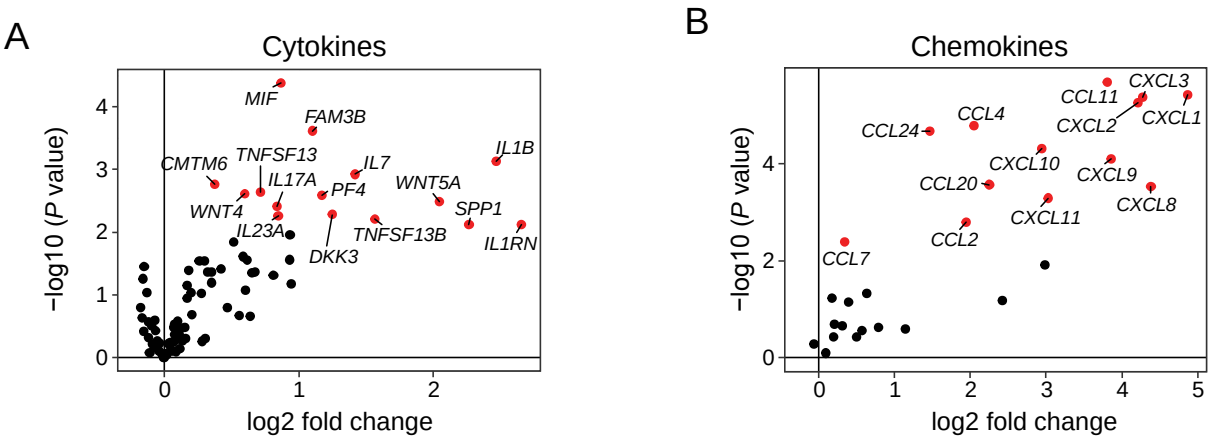

Figure 3.16. Human UC is associated with IL-1β and neutrophil-recruiting chemokines. Volcano plot showing enrichment of cytokines (A) and chemokines (B) in colonic biopsies from patients with UC versus healthy controls. Data were derived from the GEO dataset GSE9452. HC *n* = 5; UC *n* = 8. *P* values were calculated using limma with multiple correction using BH.

Having identified major cytokine and chemokine pathways associated with IBD, we sought to understand how FcγRIIA may contribute to intestinal inflammation. Correlation analysis demonstrated that *FCGR2A* expression levels in pooled healthy and UC colonic biopsies correlated with several UC-associated cytokine and chemokine gene transcripts (Fig. 3.17). Most strongly correlated were the cytokine IL-1β and the chemokines CXCL8, CXCL1 and CXCL2 (Fig. 3.17A, B), supporting a role for FcγRIIA in the regulation of these inflammatory mediators. Furthermore, mucosal *IGHG1* expression levels also correlated significantly with IL-1β and CXCL8 (Fig. 3.17C).

Secondly, we used an unbiased hierarchical clustering algorithm to investigate how a curated list of all known chemokines and cytokines correlated with *FCGR2A* expression across these same samples (Fig. 3.18). Strikingly, *FCGR2A* expression clustered most strongly to *IL1B* once more (Fig. 3.18A). This was subsequently confirmed in an independent cohort (Fig. 3.18B). Given the strong association of IL-1β production with UC (Fig. 3.16), these results suggest that FcγR signalling may be involved in the regulation of inflammation-associated IL-1β production in the GI tract.

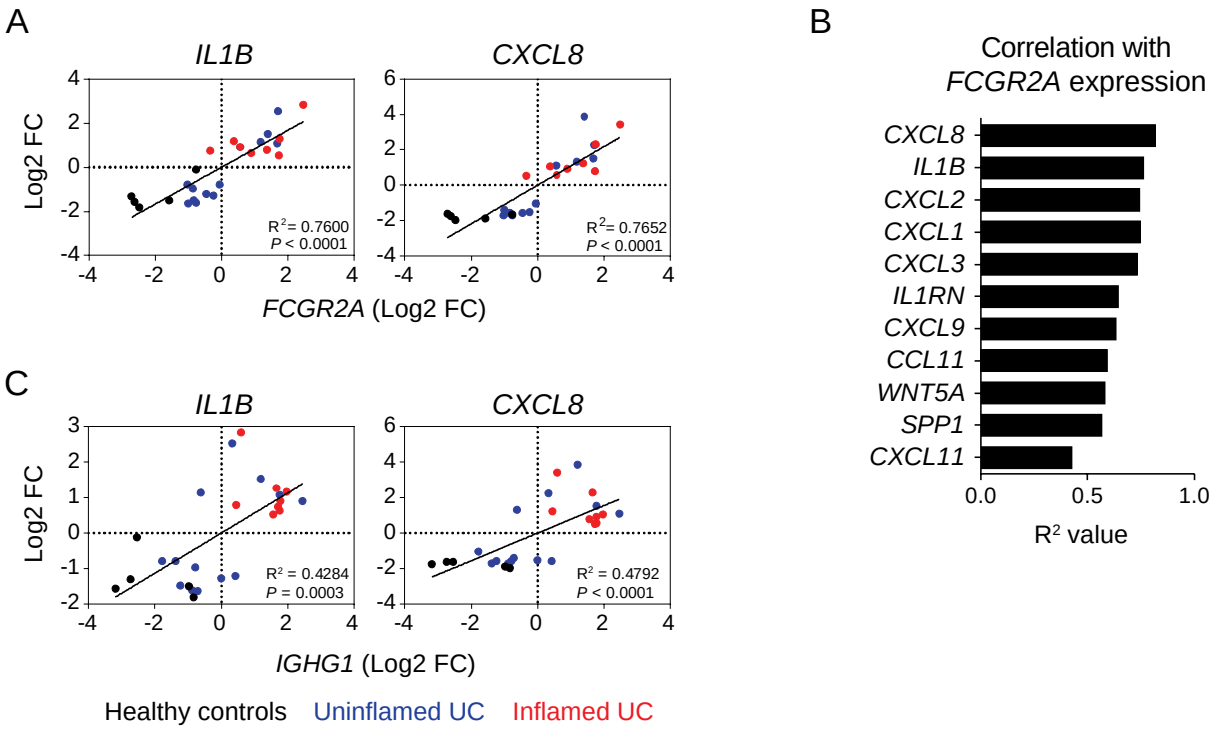

Figure 3.17. *FCGR2A* correlates with IL-1β and CXCL chemokines in UC. (A) Correlation of *FCGR2A* gene transcripts with *IL1B* and *CXCL8* in pooled healthy control (black), non-inflamed UC (blue), and inflamed UC (red) colonic biopsies. Data were derived from the GEO dataset GSE9452. (B) Summary of *FCGR2A* correlations with UC-associated cytokine and chemokine gene transcripts in pooled healthy controls, non-inflamed UC, and UC patient colonic biopsies. (C) Correlation of *IGHG1* gene transcripts with IL1B and CXCL8 gene transcripts in pooled colonic biopsies from UC patients. *P* values were calculated using linear regression analysis.

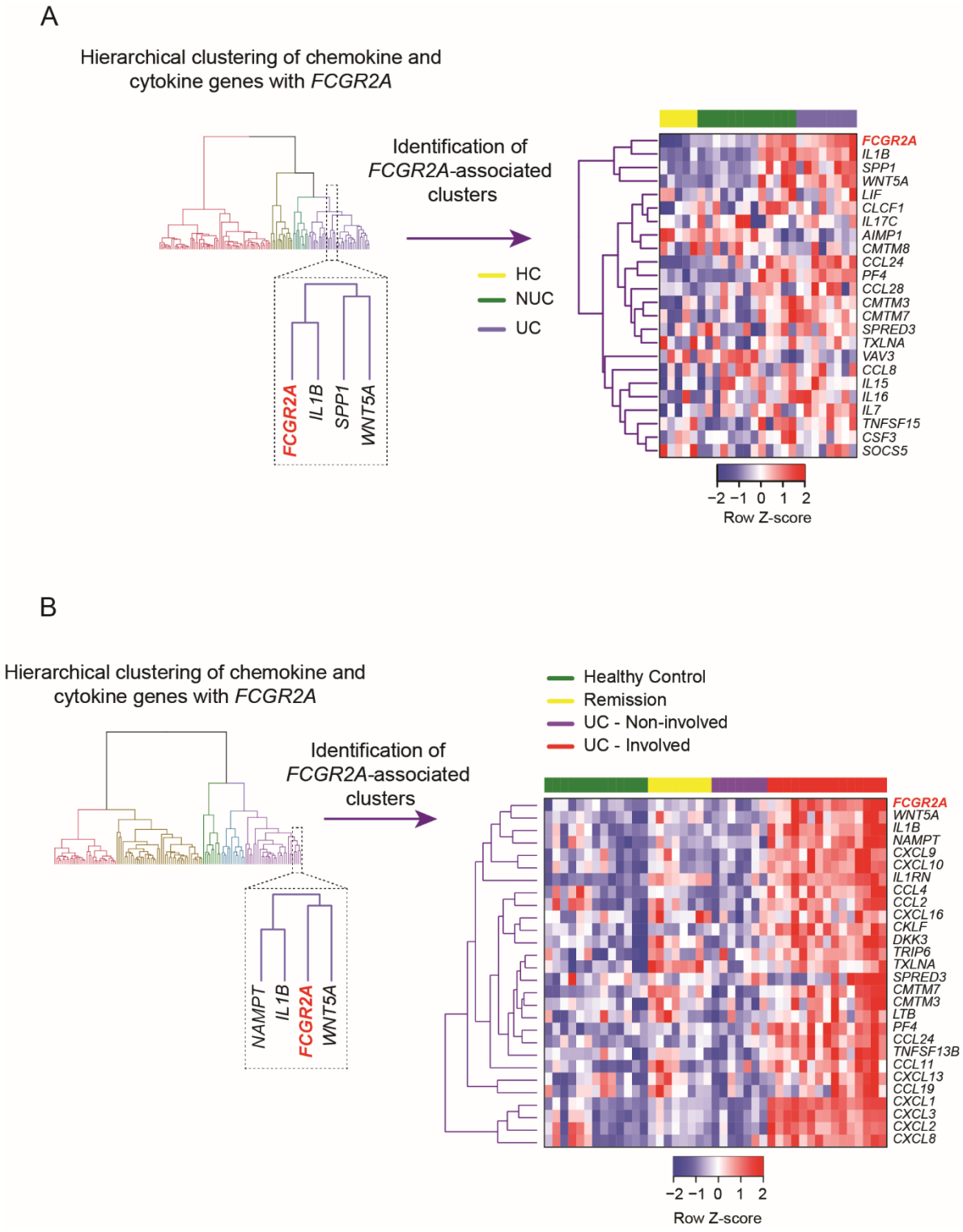

Figure 3.18. *FCGR2A* clusters consistently with *IL1B* in UC biopsies. Hierarchical clustering of chemokine and cytokine genes with *FCGR2A* expression levels in two datasets of pooled healthy controls and UC patient colonic biopsies (GSE9452 = top; GSE38713 = bottom). Complete cytokine gene list found in Table S1, Appendix.

We sought to investigate whether similar networks are also associated with chronic murine DSS-induced colitis. Transcriptomics analysis of murine colons following chronic DSS administration revealed *Il1b* as the most significantly enriched cytokine compared to healthy controls (Fig. 3.19A), while chemokine analysis once again demonstrated significant enrichment of several neutrophil-associated chemokines, including *Cxcl2* (Fig. 3.19B).

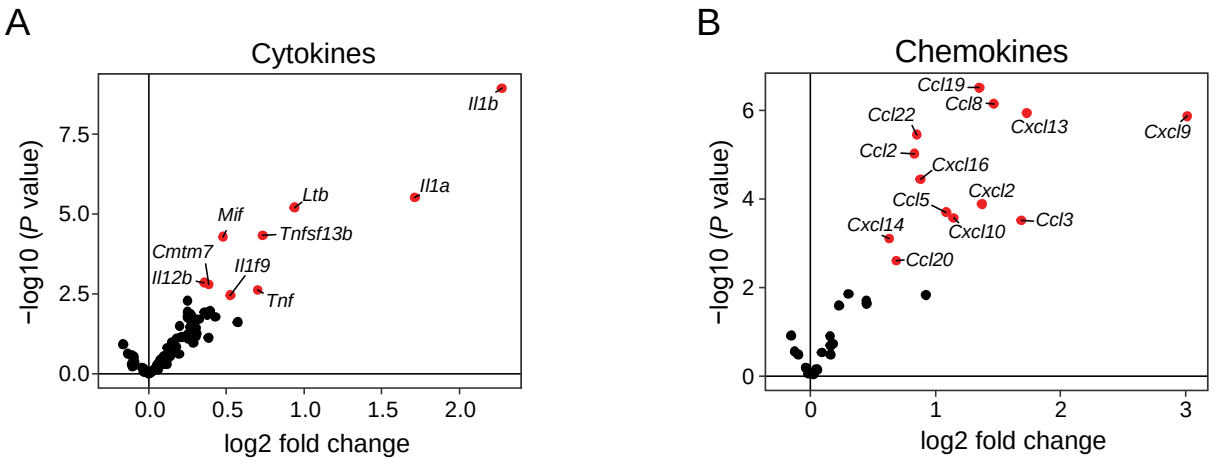

Figure 3.19. IL-1β and CXCL chemokines are associated with chronic murine colitis. Volcano plot showing enrichment of cytokines (A) and chemokines (B) in whole murine colonic tissue following chronic DSS administration versus healthy controls. Data were derived from the GEO dataset GSE42768. H_2O *n* = 5; cDSS *n* = 5. *P* values were calculated using limma with multiple correction using BH.

qPCR analysis confirmed the enrichment of numerous inflammatory cytokines in chronically inflamed murine colons. These included the macrophage-associated cytokines *Il1b*, *Il6*, *Tnf*, and *Il10* (Fig. 3.20A), as well as Th17-associated cytokine genes, such as *Il17a*, *Il22*, and *Csf2* (Fig. 3.20B). In addition to its significant enrichment, *Il1b* was also the most highly expressed inflammatory cytokine of those investigated within the inflamed colons of mice, suggesting a dominant role in driving colitis (Fig. 3.20C). Finally, analysis of neutrophil- and monocyte-recruiting chemokines demonstrated a significant increase in *Ccl2*, *Ccl8*, *Cxcl1*, and *Cxcl2* (Fig. 3.20D).

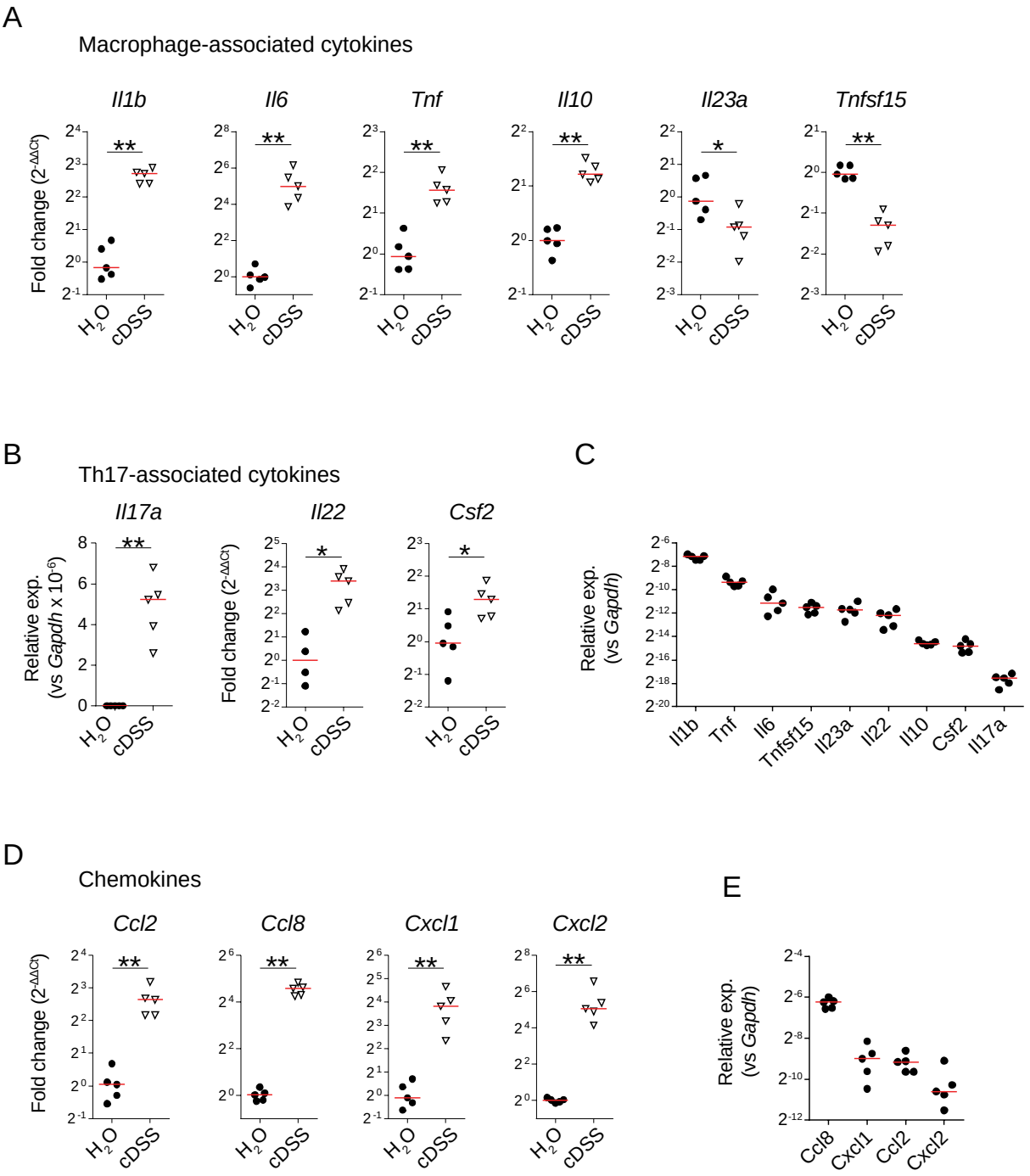

Figure 3.20. Chronic DSS administration is associated with IL-1β production, Th17 immunity, and chemokine production. qPCR of macrophage-associated cytokine genes (A), Th17-associated cytokines (B), and macrophage-derived chemokines (D) in whole colonic tissue following cDSS versus healthy controls. Relative expression of cytokines (C) and chemokines (D) in whole colonic tissue following cDSS. Data are representative of two independent experiments. *P* values were calculated using the nonparametric Mann-Whitney test. * $P < 0.05$; ** $P < 0.01$.

In agreement with human data, we observed a strong correlation between FcγRIII expression levels and several Th17 cytokines and neutrophil-recruiting chemokines (Fig. 3.21A). Strikingly, the most robust correlations were once again with CXCL1 and IL-1β gene expression levels (Fig. 3.21B).

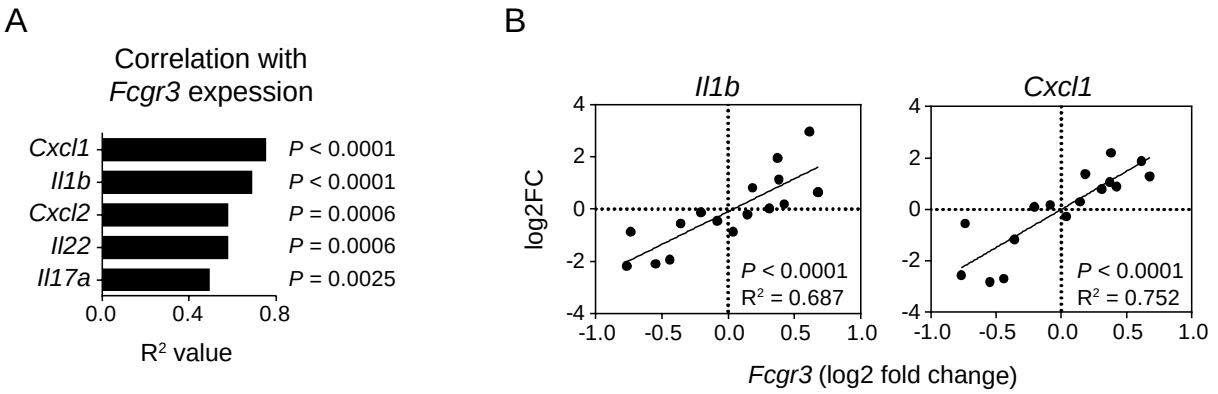

Figure 3.21. Correlation of *Fcgr3* with *Il1b* and *Cxcl1* in murine chronic colitis. (A) Correlation of *Fcgr3* expression levels with Th17-associated cytokine and chemokine gene transcripts by qPCR in whole colonic tissue following cDSS. (B) Correlation of *Fcgr3* with *Il1b* and *Cxcl1* in whole colonic tissue by qPCR. Data are representative of three independent experiments. *P* values were calculated using linear regression analysis.

Therefore, chronic DSS administration results in a pronounced increase in local IL-1β production, the induction of local Th17 immunity, and neutrophil-recruiting chemokine expression, with expression levels correlating strongly with *Fcgr3*, the murine homologue of FcγRIIA. As such, chronic DSS-induced colitis is a useful model for studying the effects of FcγR signalling in intestinal disease progression.

3.7. Immune complex stimulation of intestinal leukocytes

We have identified IL-1β as a major UC-associated cytokine and demonstrated a robust and reproducible correlation with FcγRIIA in humans and FcγRIII in mice. Furthermore, we have demonstrated that intestinal macrophages, known sources of pro-inflammatory IL-1β, express the highest levels of FcγRs within the GI tract, while *in vitro* studies have demonstrated a role for IgG in supporting IL-1β-dependent Th17 cells in co-cultures with DCs and macrophages [74], [75], [82], [279].

To investigate whether IgG may have a direct role in driving intestinal Th17 immunity via IL-1β, we firstly isolated inflamed murine LPMCs at day 14 following 2 % DSS administration and stimulated them with O or O-IC for 16 h. O-IC stimulation induced production of IL-1β, but no change in IL-23 levels, as determined by ELISA (Fig. 3.22A). Furthermore, O-IC stimulation

resulted in increased production of the key Th17 mediators IL-17A and IL-22 (Fig. 3.22B), and the neutrophil-recruiting chemokines CXCL1 and CXCL2 (Fig. 3.22C). These observations were mirrored in human LPMCs: O-IC stimulation of human ileal LPMCs resulted in increased expression of IL-1β and CXCL8 (Fig. 3.22D). Therefore, IgG potently stimulated intestinal immune cells and resulted in the induction of an inflammatory network resembling that identified by FcγR correlation analysis.

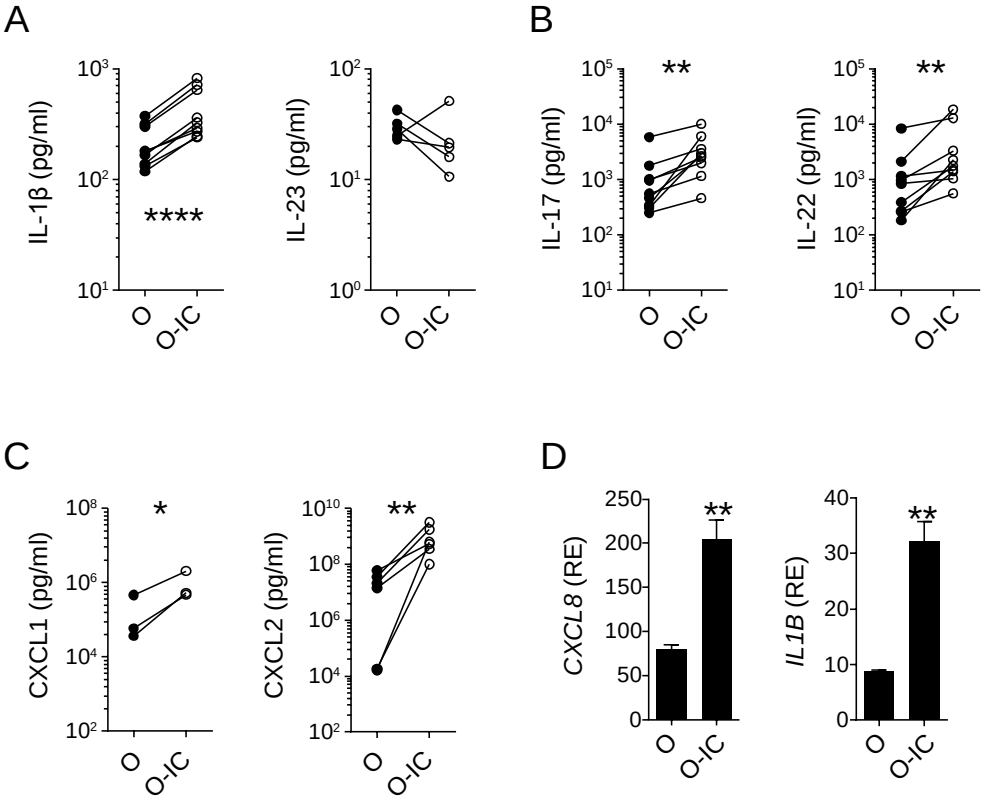

Figure 3.22. Immune complexes induce IL-1β and CXCL chemokine production by intestinal immune cells. ELISA of macrophage-associated IL-1β and IL-23 (A), Th17-associated IL-17 and IL-22 (B), and neutrophil-recruiting chemokine production by inflamed murine intestinal LPMCs stimulated with O or O-IC for 16 h. Paired samples represent cells derived from the same mouse. (D) Expression of CXCL8 and IL-1β mRNA by human LPMCs following 16 h stimulation with O or O-IC for 16 h. *n* = 3 per group. Data are representative of three independent experiments. *P* values were calculated using ratio-paired t tests (A-C) and the parametric Student's t test (D). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

To address whether anti-commensal IgG could directly drive inflammatory cytokine expression *in vivo*, anti-*E. coli* IgG antibodies were passively transferred into mice during an acute course of DSS administration. Colonic *E. coli* levels are known to increase with the onset of intestinal inflammation and exacerbate disease [305]–[308]. As such, they are likely to represent a major target of anti-commensal humoral immunity. While addition of exogenous anti-commensal IgG

did not induce significant changes in weight loss, an increase in clinical disease activity was noted (Fig. 3.23A). Furthermore, qPCR analysis of whole inflamed colonic tissue demonstrated an increase in expression of macrophage-associated cytokines, including *Il1b* and *Tnf* (Fig. 3.23A), as well as *Il17* and *Csf2* (Fig. 3.23B), and several chemokines, including *Cxcl1* and *Cxcl2* (Fig. 3.23C). Therefore, anti-commensal IgG antibodies can directly promote the inflammatory response *in vivo*.

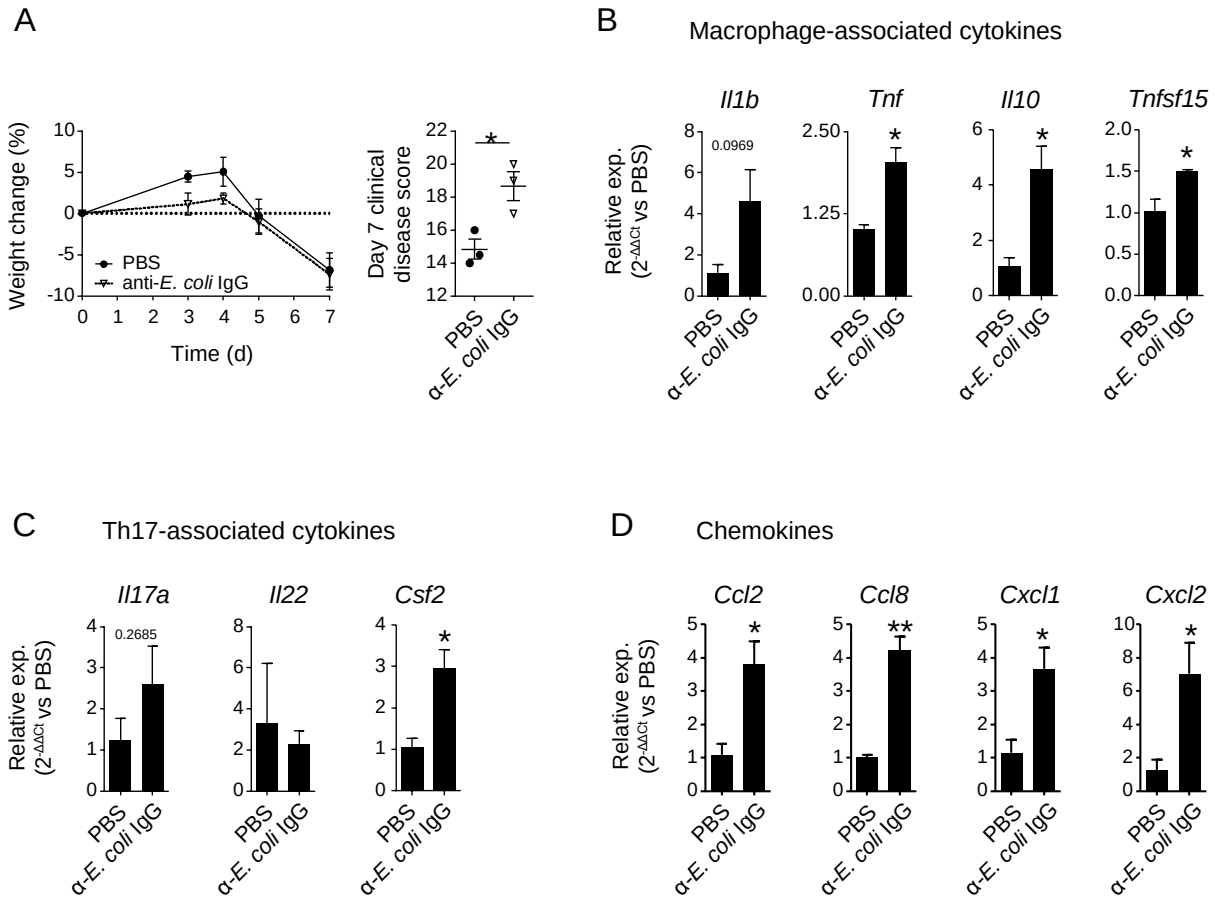

Figure 3.23. Anti-commensal IgG induces colonic inflammation and cytokine production. (A) Weight loss of WT mice treated with PBS or 1 mg anti-*E. coli* IgG (i.p.) at day 0 and day 3 of 7-day acute DSS protocol. *n* = 3 per group. Clinical disease score calculated as described in Table 2.3. (B-D) qPCR of macrophage-associated cytokines (B), Th17-associated cytokines (C), and macrophage-derived chemokines (D) in whole inflamed murine colonic tissue following injection of anti-E. coli IgG or PBS and acute DSS administration. *n* = 3 per group. Data are representative of two independent experiments. *P* values were calculated using the parametric Student's *t* test. * $P < 0.05$; ** $P < 0.01$.

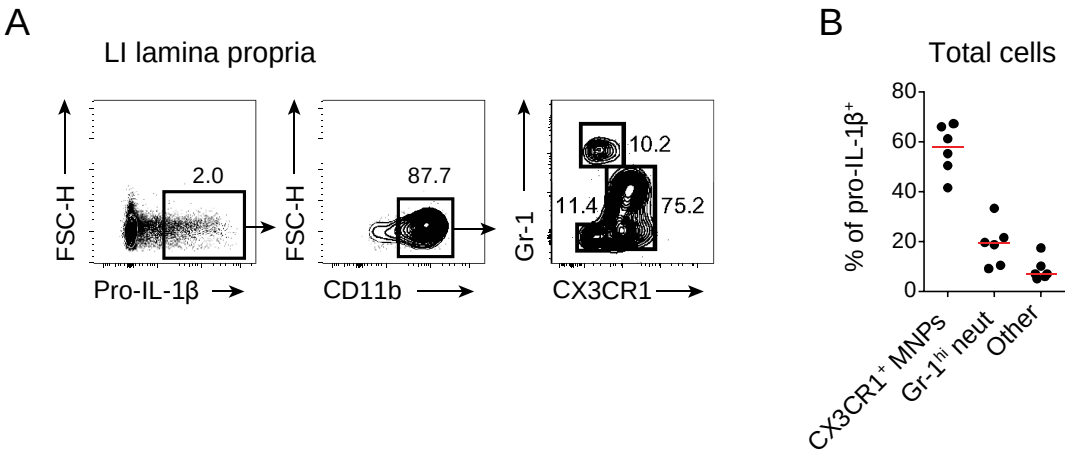

Figure 3.24. CX3CR1^{+} MNPs are the major source of colonic IL-1β production in inflammation. (A) Flow cytometry of pro-IL-1β-producing subsets in the inflamed colonic murine lamina propria. (B) Quantification of pro-IL-1β^{+} subsets in A. Data are representative of three independent experiments.

Tissue-resident macrophages and monocytes are known to be potent producers of intestinal IL-1β, and we hypothesised that they would be the major IL-1β source in these cultures. This was confirmed by flow cytometry, with minor contributions from neutrophils and dendritic cells (Fig. 3.24A, B).

3.8. Discussion

3.8.1. Anti-commensal IgG is increased in UC and correlates with disease activity

IgA is the predominant immunoglobulin isotype in the GI tract during homeostatic conditions, playing key roles in the maintenance of host-commensal mutualism through immune exclusion [42]. Recent seminal studies, however, have demonstrated that anti-commensal IgG forms part of normal adult immunity, with consequences for vertical transfer of immunity to neonates, immune cell development, and resistance to systemic infection [477], [479], [616]. This IgG is thought to be largely TCR-dependent and TI, suggesting a non-GC origin.

Under inflammatory conditions, such as infection and IBD, an increase in local and systemic IgG is observed in humans and mice [283], [430], [482]. However, little is known about GI IgG subclass distribution, antigen-specificity, FcγR binding capacity, or glycosylation status, creating a void in our understanding of how mucosal IgG contributes to inflammation.

Here, we sought to investigate the nature of the IgA and IgG response in patients with UC. We demonstrated a significant enriched of *IGHM*, *IGHD*, and *IGHG1* transcripts in UC biopsies compared to controls, with little change in *IGHA*. Although only a small cohort of patients, here we observed a significant increase in total IgG-opsonised luminal bacteria in patients with UC compared to HHCs, which correlated with disease activity: patients with a Wormsley CAI over 10 exhibited the highest levels of IgG-bound luminal bacteria. Once more, no significant difference in IgA1/2 opsonisation was observed between HHCs and UC patients, consistent with transcriptomics data. Further stool sample collection would help to increase the power of these observations, and is currently in progress.

Unfortunately, IgG subclasses were not investigated here, and remains an open avenue of investigation. Due to sequence similarity amongst *IGH* transcripts, specific probes to all *IGH* gene transcripts are not available in transcriptomics datasets. As such, it is not possible to analyse the distribution of IgG subclasses in this manner. Nonetheless, these observations have several implications. IgG1 in humans, along with IgG3, is potently pro-inflammatory and dominates adaptive humoral immune responses to protein antigens. IgG1 can activate complement and bind FcγRs with higher affinity that IgG2 and IgG4, suggesting that the local engagement of inflammatory mechanisms is likely in UC following IgG generation. Increases in *IGHM* and *IGHD*, the latter not contributing to the secreted immunoglobulin repertoire, likely reflect a combination of increased immune IgM secretion and B cell infiltration into the inflamed mucosa. Therefore, these results suggest that B dynamics and isotype distribution of Ig is significantly altered during IBD, with a switch away from non-inflammatory IgA to inflammatory IgG, including IgG1.

We observed a striking association between the extent of IgG opsonisation and disease activity. Whether IgG subclass distribution is associated with differential pathology is of particular interest: IgG1 and IgG3 could contribute to enhanced inflammatory responses relative to IgG2 and IgG4. For example, could individuals with high IgG titre but lower disease activity be associated with an enrichment of IgG2 and IgG4? It should be noted that of those individuals investigated, patients with high IgG titres consistently had a high CAI score, while individuals with little IgG had low disease activity. Of particular interest are those UC patients that exhibit intermediate IgG levels and differential disease progression/prognosis. As well as subclass, IgG glycosylation profile may affect disease management. Indeed, a systems serology approach in IBD may help to clarify some of these observations.

Whether IgG is a driver of inflammation or simply a surrogate of the inflammatory state is not clear from these results. However, it seems possible that they go hand-in-hand. Local disease activity is a function of inflammatory cytokine production in the gut, with IL-1β, IL-23, and Th17 immunity successfully targeted in IBD therapeutics [364], [365]. Th17 immunity has been implicated in class-switching to IgG subclasses, while an IL-23/Th17 axis has been demonstrated to promote desialylation of IgG in mice, augmenting its pro-inflammatory capacity *in vivo* in models of RA [178]. Therefore, exacerbated type 17 immune responses may contribute to the emergence of local IgG and the induction of a pro-inflammatory glycome. Furthermore, several studies have shown that IgG may potently activate Th17-inducing inflammatory profiles in human M2 macrophages and DCs [74], [611]. As such, IgG-mediated FcγR signalling may then promote a positive feedback loop in which Th17 immune responses are augmented, leading to severe detrimental inflammation.

3.8.2. Anti-commensal IgG is increased in murine DSS-induced colitis

Several murine models of IBD exist, as discussed in Chapter 1. However, the majority of these models are not appropriate for the investigation of B cells. T cell-transfer and anti-CD40 monoclonal antibody models rely on the use of immunodeficient mice lacking T or B cells [299], [303]. Furthermore, *H. hepaticus* infection is often carried out in similarly immunocompromised strains [298]. This has led to the general assumption that B cells are not involved in the pathogenesis of chronic inflammatory disorders of the GI tract. This seems surprising, given that sterilising immunity to *C. rodentium*, a widely used model of human A/E *E. coli* infection, is critically dependent on B cells and IgG [283], [284], [473]. This model has yielded invaluable insights into basic immunological mechanisms of inflammation that have proved useful to the understanding of IBD, including the involvement of IL-23 and Th17 cells [310], [617]–[619], and support the notion that IgG may be pathogenic. Similarly, anti-flagellin IgG transfer has been shown to directly promote DSS-induced colitis [482].

Further considerations revolve around the nature of the immune response elicited in distinct models. While *C. rodentium* infection can recapitulate analogous inflammatory mechanisms to IBD, it does not induce the overwhelming inflammation associated with human disease. We settled on the use of the colitogen DSS. As DSS acts to erode the epithelial monolayer, it mirrors defects in epithelial barrier integrity and disease pathology associated with UC. This is of particular importance, given the stronger association of FcγRIIA-H131 with UC over CD. Furthermore, this model is dependent on the microbiota, can be used for acute or chronic colitis, induces inflammation in immunocompetent mice, and has been associated with IgG generation in the past.

In agreement with a previous study [482], we demonstrated that DSS administration induced *de novo* serum anti-flagellin and bulk anti-commensal IgG antibodies. Unlike other studies, we also demonstrated significant enrichment of IgG within the inflamed colonic lumen and binding to luminal commensal microbes. The effect of DSS administration on IgG opsonisation was smaller than the differences observed in UC patients compared to HHCs. However, the acute nature of this model (only 7 days) likely underlies these observations. Nonetheless, DSS-induced colitis is a reasonable model for the investigation of IgG-driven inflammation in the gut.

3.8.3. IgG$^+$ B cells are increased in the inflamed murine colon

We hypothesised that the increased IgG response may result from gut-infiltrating B cells. Consistent with this, we observed a significant influx of CD19$^+$ B220$^+$ B cells into the inflamed colonic mucosa of mice following chronic DSS administration, and a skewing to from IgA to IgG. This was confirmed by confocal microscopy. Surprisingly, B cells made up the major adaptive lymphocyte population in this setting and demonstrates a considerable aspect of mucosal immunity that is omitted from several murine models of colitis.

The nature of this B cell response remains unknown. In line with the study by Koch et al., inflammation-associated IgG may arise in a TI, TLR-driven manner within the GALTs or intestinal LP itself [477]. Whether APRIL and BAFF are significantly induced in the gut following DSS has not been investigated here, but may contribute to the formation of local niches for IgG plasma cell survival or local CSR, particularly at early timepoints following DSS administration. However, high-affinity IgA responses are known to be induced in response to pathogens. Given the inflammatory milieu induced by DSS administration, it seems likely that otherwise low-affinity IgA-inducing microorganisms may induce higher affinity Ig responses during colitis as a result of secondary signals and increased bacterial penetration beyond the epithelial barrier. One might expect these high affinity IgG clones to arise later in inflammation following rounds of SHM and selection within GALT GCs. A systematic characterisation of

CSR-associated signals that are differentially induced during chronic colitis versus healthy controls are lacking, both here and in the literature.

3.8.4. FcγR signalling is enriched in intestinal inflammation

FcγR signalling is critically implicated in UC pathogenesis and protection against *C. rodentium* infection [523]. These results raise the possibility that FcγRs can contribute to inflammatory mechanisms in the GI tract. Following the observed increase in local IgG and microbial opsonisation in intestinal inflammation, we decided to characterise the FcγR expression and signalling profile in UC patients and DSS-induced colitis.

We demonstrated that FcγR transcripts are enriched in UC colonic biopsies, with a smaller enrichment of FcγRIIB gene transcripts relative to the activating FcγRs. As such, global FcγR A/I ratios are skewed towards activation. This was confirmed in inflamed murine colonic tissue, and GSEA demonstrated that FcγR signalling pathways, as well as FcγR genes themselves, are enriched in UC and DSS-induced colitis. Furthermore, FcγR signalling was associated with severe inflammation, with FcγR transcript enrichment, elevated global A/I ratios, and enriched FcγR signalling pathways in infliximab-refractory IBD biopsies. These observations were supported by characterisation of FcγR expression profiles on gut-infiltrating cells in mice, demonstrating enrichment of FcγR-expressing macrophages and neutrophils, as well as increased macrophage A/I ratios during colitis relative to healthy mice. Whether or not enhanced FcγR signalling in infliximab-resistant IBD is reflective of a generalised exacerbated inflammatory response or a bone fide mechanism of resistance is to be elucidated, but demonstrates a strong association between this pathway and colitis.

Macrophages were of particular interest. These cells expressed the highest levels of FcγRs, are known to predominantly reside within the LP, and directly sample luminal contents. Therefore, during inflammation, these cells are perfectly positioned to interact with IgG-opsonised bacteria that may penetrate the damaged epithelium. Indeed, macrophage infiltration is common during various inflammatory disorders, including colitis. In this setting, macrophages have been shown to drive intestinal inflammation through production of TNF, IL-1β, and IL-23, as demonstrated in humans and several murine models [82], [279], [295], [511]. These cells also orchestrate the recruitment of immune cells through the production of chemokines, including CXCL1 and 2, and CCL8. Our results demonstrate that tissue-resident macrophages upregulate expression of activating FcγRs during inflammation, with implications for FcγR-driven inflammatory responses. In this way, the intestinal immune system is poised to respond to local IgG during inflammation, which we previously demonstrated to include significant IgG1 production. How FcγR expression is regulated is not clear, but may include IFNγ secretion: DSS administration is known to induce colonic IFNγ production which can drive intestinal inflammation [301], [393]. IFNγ can downregulate expression of FcγRIIB and induce

activating FcγR expression [84]. Furthermore, PGE2 has been shown to have important roles in the activation of intestinal immune cells, such as ILC3s [544], and can modulate FcγR expression on human monocytes. Therefore, it is likely the several factors in the GI milieu contribute to this differential FcγR expression during colitis. Despite its association with many immune cells, including B cells and DCs, macrophage expression of FcγRIIB was strikingly higher than other immune populations in the gut. This raises the possibility of a relatively targeted macrophage response by modulating FcγRIIB expression *in vivo*.

It is of interest whether these mechanisms act in concert with IgG CSR. For example, $CD8^+$ T cells were shown to induce IgG CSR in mice following adoptive transfer via an IFNγ-dependent mechanism. Therefore, IFNγ could potentially promote local IgG generation and the modulate of FcγR-expressing cells for enhanced sensitivity to IgG in a cooperative manner.

As well as in mice, we demonstrated that human ileal macrophages also express high levels of FcγRs, in particular FcγRIIA and FcγRIIB. Therefore, this raises the possibility that FcγRIIA polymorphisms directly influence the activity of these cells, and macrophage-mediated inflammatory mechanisms may underlie the association with FcγRIIA-H/R131 and IBD susceptibility. Whether FcγR A/I ratios similarly change in human IBD have not been investigated here, but are supported by the observations of enhanced global A/I ratios within the inflamed GI tract.

3.8.5. FcγR signalling is associated with IL-1β production in humans and mice

Several inflammatory cytokine pathways are associated with IBD, which can cause some confusion. This includes exacerbated Th17 responses in both CD and UC, elevated IL-13 production in UC, and IFNγ production in CD [318]–[320]. Therefore, IC signalling could potentially influence a variety of inflammatory pathways. In order to tackle this problem, we took an unbiased approach to understanding predominant UC-associated pathological pathways and their association with FcγR signalling.

We demonstrated elevated IL-1β expression in UC and a strong correlation with FcγRIIA expression levels. Strikingly, this association was confirmed by hierarchical clustering algorithms investigating FcγRIIA correlation with all cytokines and chemokines in two independent UC datasets. IL-1β expression was also characteristic of chronic DSS-induced colitis and strongly associated with FcγRIII expression, the murine homologue of FcγRIIA.

These results firstly demonstrate that IL-1β is a major inflammatory cytokine in both human and murine colitis, and is consistent with several studies that have demonstrated therapeutic benefit to IL-1β blockade or inflammasome deficiency in chronic intestinal pathology [279]. Furthermore, these results support the hypothesis that FcγR signalling may regulate Th17 immunity in the gut through production of several upstream cytokines. It is notable that, while

elevated in UC biopsies, IL-23A expression was not significantly correlated with FcγR expression or with chronic murine colitis. IL-1β has roles in the systemic inflammatory response, neutrophil recruitment to tissues, and in the activation of Th17 immunity. Its role in the latter is of particular interest here: in the presence of IL-1β, IL-23-induced Th17 cells exhibit an elevated inflammatory profile, with T-bet and IL-33 expression [352]. Therefore, FcγR signalling may act to potentiate mucosal Th17 responses initiated by IL-23 production through an IL-1β-dependent mechanism. This is consistent with a study by Uo et al., which demonstrated that IL-23 expression in intestinal macrophages was refractory to FcγR stimulation, and the previously established link between FcγR signalling and IL-17A production in a model of fatal glomerulonephritis [183], [430].

Care should be taken at this point not to over-interpret these results, however. Correlation may simply be reflective of a broader inflammatory network, in which both IL-1β and FcγR expression is increased. However, the tight, reproducible association suggests these proteins may be at least expressed by the same cells, and regulated in unison.

3.8.6. Immune complexes induce IL-1β and Th17-associated cytokine production by intestinal immune cells

To address this association more directly, LPMCs were isolated from the inflamed colonic LP following DSS administration or the human ileum and stimulated with IC. IL-1β was consistently induced in response to IC stimulation in both mice and human LPMCs, while IL-23 was refractory to stimulation. Furthermore, IL-17 and IL-22 production was significantly increased, Th17 cytokines downstream of IL-1β.

These results support previous associations between FcγR signalling and IL-1β, but not IL-23, and demonstrate that IgG signalling is sufficient to promote Th17 inflammatory pathways *in vitro*. It is tempting to speculate that this increase in IL-17 and IL-22 is specifically IL-1β-dependent, but we cannot rule out the induction of IL-1β/IL-23-independent inflammatory cytokines that may be induced following IC. This includes PGE2 and TL1A, both of which have been shown to be induced by IC signalling on macrophages [79], [612].

Consistent with these results, we demonstrated that passive transfer of anti-*E. coli* IgG into mice during DSS-induced colitis augmented inflammatory cytokine production within the mucosa, including IL-1β, TNF, IL-10, TL1A and several chemokines. Outgrowth of *E. coli* is characteristic of inflammation, including in DSS-induced colitis [305]–[308]. Therefore, anti-*E. coli* IgG likely represents a reasonable model antigen that would be present in patients with IBD and in murine models. It is noteworthy that this passive transfer did not induce significant IL-17 or IL-22 production, but elevated GM-CSF levels. This may result from the 7-day nature of the protocol in this instance: T cell-derived IL-17 and IL-22 may require a more chronic exposure to DSS in order to induce adaptive Th17 immunity. However, chronic DSS exposure

would confound the results of the passive transfer, as endogenous IgG would be generated *de novo* beyond this point.

3.8.7. Summary

Here, we have demonstrated the emergence of an anti-commensal IgG response during intestinal inflammation and its association with an IL-1β-mediated Th17 response (Fig. 3.25). Macrophages were identified as the major source of IL-1β in the inflamed colon. Therefore, these results raise the possibility that macrophage-intrinsic FcγR signalling may drive IL-1β production and the induction of type 17 immunity. This hypothesis is consistent with previous studies in humans, demonstrating that TLR-FcγR co-stimulation on M2 MDMs induces IL-1β production, but is contrary to reports on murine macrophages that suggest FcγR-induced PGE2 production suppresses LPS-induced IL-1β production *in vitro* [74], [611], [612]. It should be noted that PGE2 is also known for its ability to support Th17 immunity [544], and so its effects may be heavily context dependent. This is also true of macrophages: while M2 TLR-FcγR co-stimulation induces IL-1β, this is not the case for M1 macrophages. Therefore, care must be taken in extrapolating *in vitro* observations to biological effects *in vivo*. The macrophage dynamic within the GI tract is complex. Intestinal macrophages resemble a mixed M1/M2 phenotype, with IL-10 and TNF production, while IL-1β is potently induced during colitis. Therefore, FcγR signalling could have differential effects on macrophages under homeostatic and inflammatory conditions. This is further confounded by the complex canonical and non-canonical caspase-mediated processing of pro-IL-1β, which may be subject to regulation by FcγR signalling directly or indirectly through secondary mediators.

Given these observations, we sought to investigate the effect of macrophage-intrinsic FcγR signalling on the production of inflammatory mediators that may contribute to intestinal inflammation. Of particular interest was the effect of IC on IL-1β, given its association with human and murine colitis, and its upregulated production by LPMCs following IgG stimulation. Furthermore, we sought to investigate the effects of FcγR signalling *in vivo* through the use of murine models in which the A/I ratio of macrophages was altered. *Fcgr2b*-deficient mice lack the inhibitory FcγR on all cells and are characterised by exacerbated inflammation in murine models of infection and autoimmunity [62], [180], [183], [206]. In contrast, M-TG mice exhibit specific overexpression of FcγRIIB on macrophages, driven by the human CD68 promoter, and are protected from overwhelming inflammation and IgG-driven pathology [205]. We hypothesised that *Fcgr2b*-deficient mice would exhibit worse pathology during chronic DSS-induced colitis, driven by exacerbated pro-inflammatory cytokine and chemokine responses, while M-TG mice would be protected. These results are discussed in the next chapter.

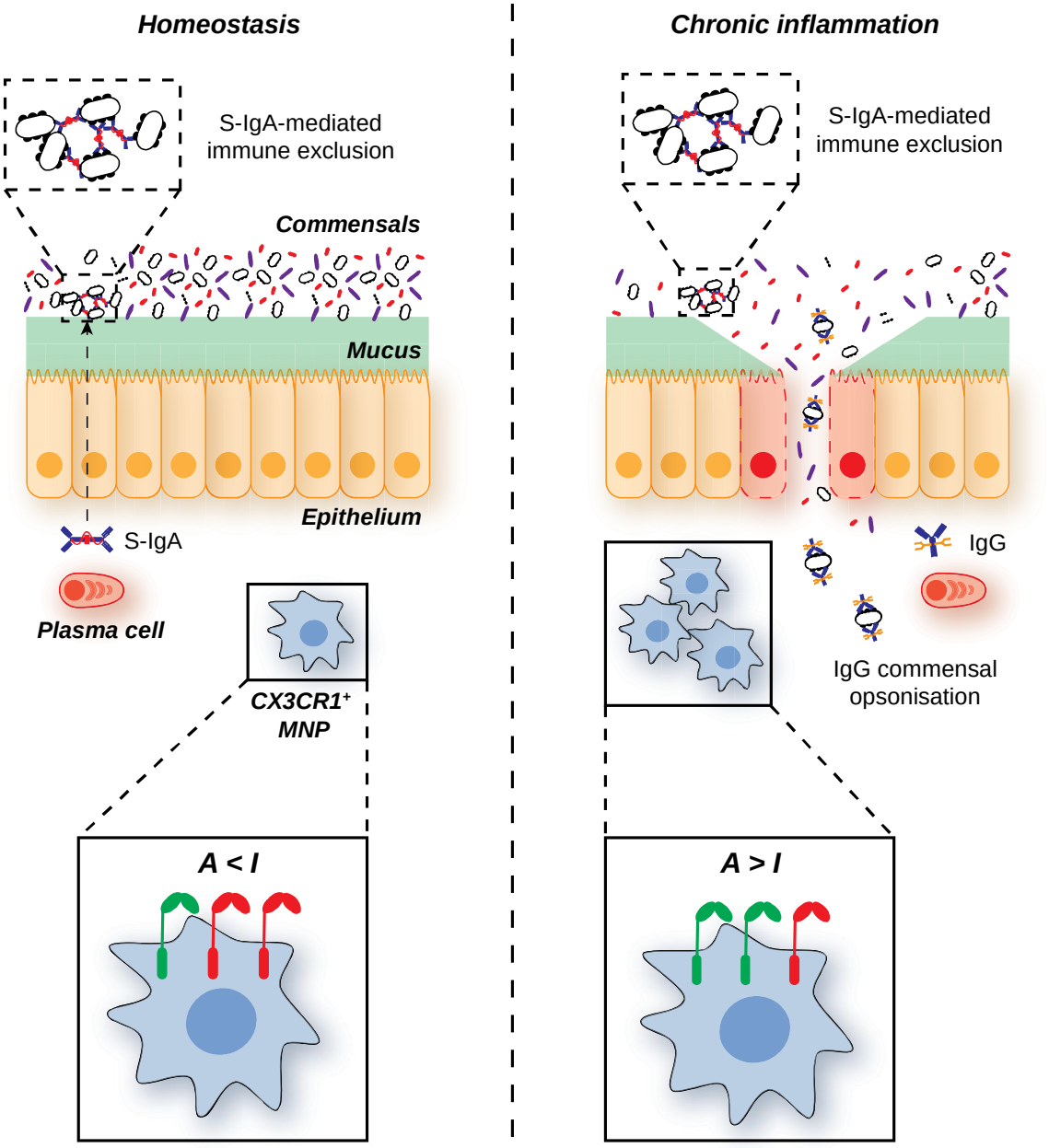

Figure 3.25. Chapter 3 graphical summary. During homeostasis, IgA is the predominant Ig isotype in the GI tract, where it is transported across the epithelium and functions via immune exclusion. During chronic inflammatory conditions, such as UC and DSS-induced colitis in mice, an anti-commensal IgG response is induced that results in significant microbial opsonisation within the lumen. FcγRIIA, a canonical activating FcγR in humans associated with UC susceptibility, and FcγRIII in mice correlate strongly with IL-1β within the inflamed colonic mucosa. Furthermore, IgG IC stimulation results in IL-1β production by intestinal immune cells in both mice and humans. As macrophages express the highest levels of FcγRs, have A/I ratios skewed towards activation during inflammation, and produce high amounts of IL-1β, these results lead us to hypothesise that macrophage-intrinsic FcγR signalling may drive IL-1β-dependent Th17 immunity and inflammation *in vivo*, directly contributing to intestinal pathology. Green = activating FcγR; red = inhibitory FcγRIIB.

4. Fcγ receptor signalling drives IL-1β-dependent intestinal inflammation

4.1. Introduction and hypotheses

We previously identified an increase in mucosal IgG responses in murine colitis and UC, reflective of an increase in LP-infiltrating IgG-producing plasma cells, as well as an enrichment of FcγR signalling pathways in UC and infliximab-refractory UC. FcγR expression was tightly associated with IL-1β and neutrophil-recruiting chemokine production, with highly significant correlations between *FCGR2A* and *Fcgr3* with IL-1β expression levels in humans and mice, respectively. IL-1β and chemokine production was directly regulated by FcγR signalling, as demonstrated by increased cytokine and chemokine expression in IgG-IC-stimulated LPMCs, as well as in response to passive transfer of anti-*E. coli* IgG antibodies during DSS-induced colitis. With a focus, specifically on IL-1β, we identified macrophages as the major source of this cytokine *in vivo*.

Next, we hypothesised that IgG-induced IL-1β production by intestinal macrophages can drive intestinal inflammation in human IBD and in murine models of colitis. Several studies have demonstrated that modulation of FcγR expression *in vivo* through the use of transgenic mice is sufficient to alter the progression of IgG-mediated disorders, with the absence of the inhibitory receptor, FcγRIIB, conferring increased disease severity in several murine models, while SNPs in human FcγR genes alter susceptibility to numerous autoimmune and inflammatory disorders [144], [161]–[163], [180], [206]. Therefore, we also hypothesised that modulation of the A/I ratio *in vivo* through the use of FcγRIIB-transgenic mice could be sufficient to alter disease severity in DSS-induced colitis, potentially mirroring the effect of *FCGR2A* polymorphisms on IBD susceptibility.

4.2. Transcriptomic analysis of FcγR signalling in intestinal macrophages

Tissue-resident intestinal macrophages, which express high levels of FcγRs, are known to be a major source of the inflammatory cytokines induced by FcγR signalling. We therefore hypothesised that direct FcγR signalling on intestinal macrophages may drive the production of inflammatory cytokines, including IL-1β and the neutrophil-recruiting chemokines CXCL1 and CXCL2.

To first address this question, we performed transcriptomics analysis on BMDMs stimulated with O-IC for 4 h to identify upregulated cytokines that were previously shown to be associated with UC (Fig. 4.1A). O-IC stimulation resulted in expression of *Il1b*, *Il1a*, *Tnf*, and *Cxcl1*. Unfortunately, *Cxcl2* did not pass quality control in this dataset, and so was absent from the analysis. We confirmed these observations at the protein level, with O-IC stimulation alone resulting in low level production of IL-1β, and production of CXCL1 and CXCL2 (Fig. 4.1B).

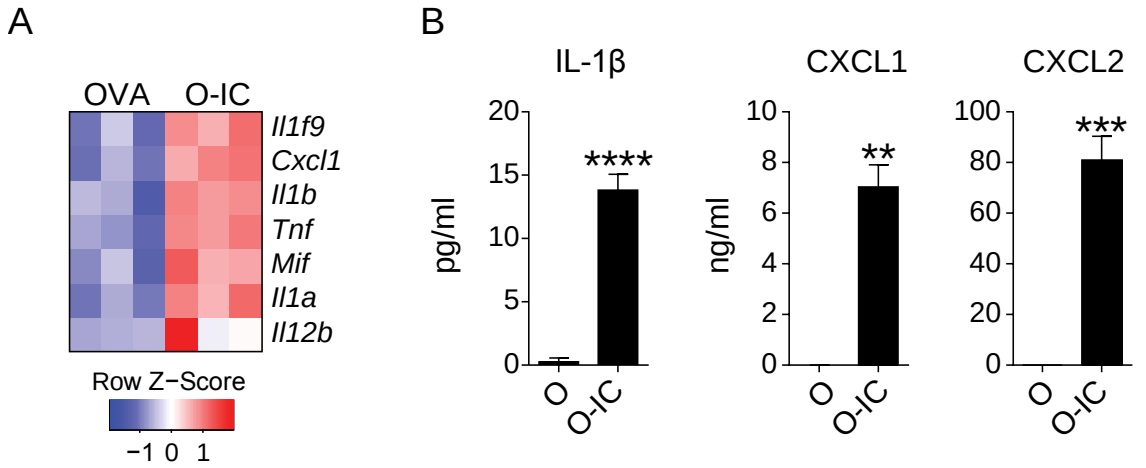

Figure 4.1. Immune complexes induce IL-1β, CXCL1 and CXCL2 production by BMDMs. (A) Heatmap of UC-associated cytokines and chemokines in BMDMs following IC stimulation. (B) ELISA of IL-1β, CXCL1 and CXCL2 protein production by BMDMs stimulated with O or O-IC for 16 h. $n = 3$ per group. Data are representative of three independent experiments. *P* values were calculated using the parametric Student's *t* test. ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Whilst this data is encouraging, BMDMs do not fully recapitulate the phenotype of intestinal macrophages. Furthermore, macrophages are known to undergo reprogramming under different inflammation conditions (e.g. M1 versus M2 macrophages) and, as such, may potentially respond differently to FcγR ligation in an inflamed colon. We therefore sought to investigate the effect of IgG IC stimulation on flow-sorted colonic CX3CR1^{+} Ly6C^{lo} MHC-II^{hi} macrophages isolated from uninflamed and DSS-inflamed mice by RNAseq. Principle component analysis (PCA) of IC-stimulated macrophages demonstrated that macrophage samples clustered based on source (i.e. inflamed or uninflamed colon) and whether they had been stimulated or not with O-IC (Fig. 4.2A). IC induced large-scale transcriptional changes

in both sets of macrophages, demonstrating IgG as a potent stimulus for tissue-resident macrophages (Fig. 4.2B).

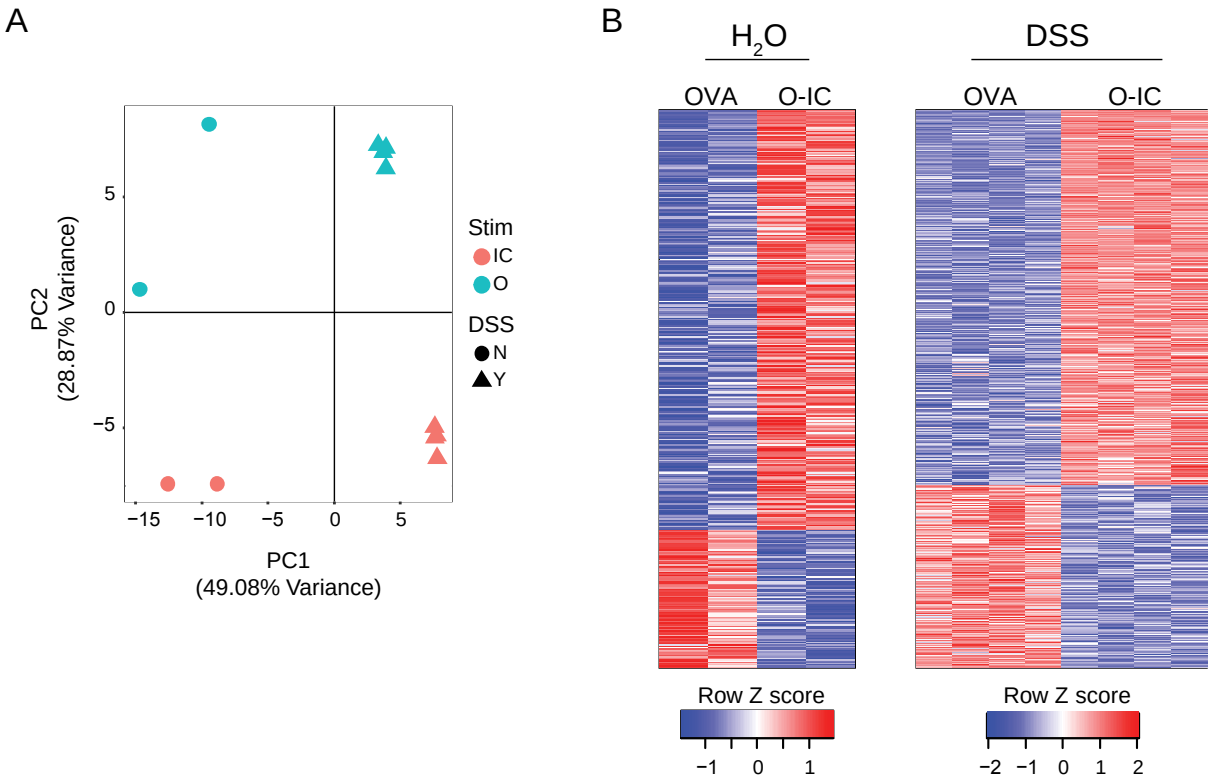

Figure 4.2. Immune complexes induce profound stimulation of intestinal macrophages. (A) PCA plot of intestinal macrophages flow sorted from uninflamed (circles) or DSS-inflamed (triangles) colons stimulated with O (turquoise) or O-IC (red) for 4 h. (B) Heatmap showing all differentially expressed genes in uninflamed (left) or DSS-inflamed (right) macrophages stimulated with O or O-IC.

Analysis of top differentially expressed genes in both sets of macrophages demonstrated large changes to several immune-associated genes, including *Cxcl1*, *Cxcl2*, *Il10*, *Ptgs2* (the gene encoding Cox2), and *Inhba*, the subunit of Activin and Inhibin (Fig. 4.3A). The general effect of IC stimulation was largely independent of whether macrophages were isolated from uninflamed or inflamed mice, although the magnitude of IC-induced changes was variable (Fig. 4.3B). One notable exception, however, was IL-6: IC stimulation resulted in increased IL-6 expression in inflamed macrophages, while it had the opposite effect on uninflamed macrophages. Given the largely similar nature of IC stimulation, and inflamed macrophages more likely representing the cells that would encounter IgG *in vivo*, we decided to focus our analysis on those macrophages isolated from DSS-inflamed colons.

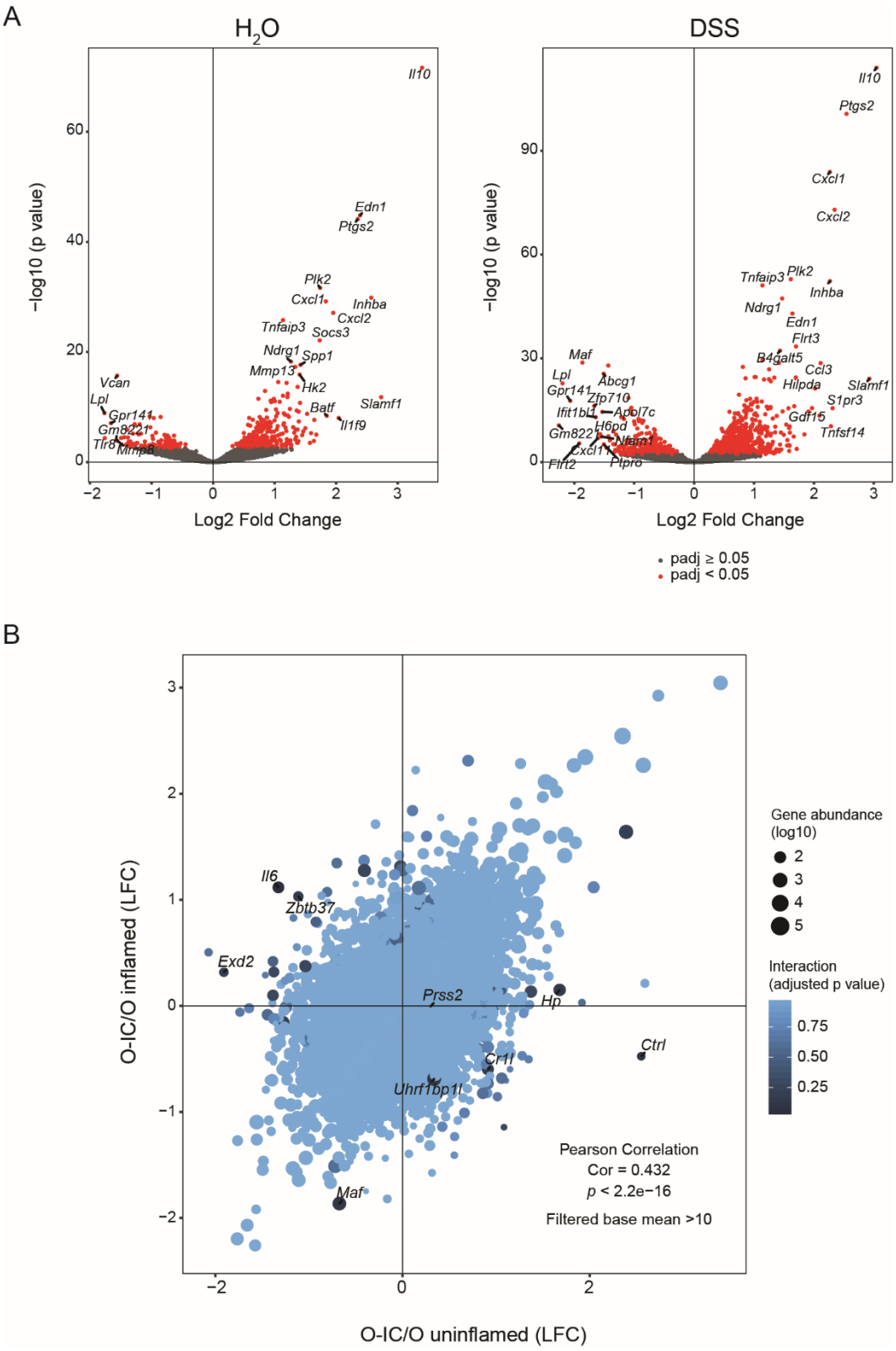

Figure 4.3. Immune complex-induced transcriptional changes are independent of inflammation state. (A) Volcano plot showing O-IC-induced transcriptional changes in colonic macrophages flow-sorted from uninflamed (left) and DSS-inflamed (right) colons. (B) Comparison of O/O-IC uninflamed and inflamed macrophages. *P* values were calculated using the standard DESeq 2 method with multiple correction using BH.

GSEA of Hallmarks pathways demonstrated that IC stimulation had a widespread inflammatory effect on macrophages, with several significantly enriched pathways, including *TNFA SIGNALLING*, *INFLAMMATORY RESPONSE*, and *HYPOXIA* (Fig. 4.4).

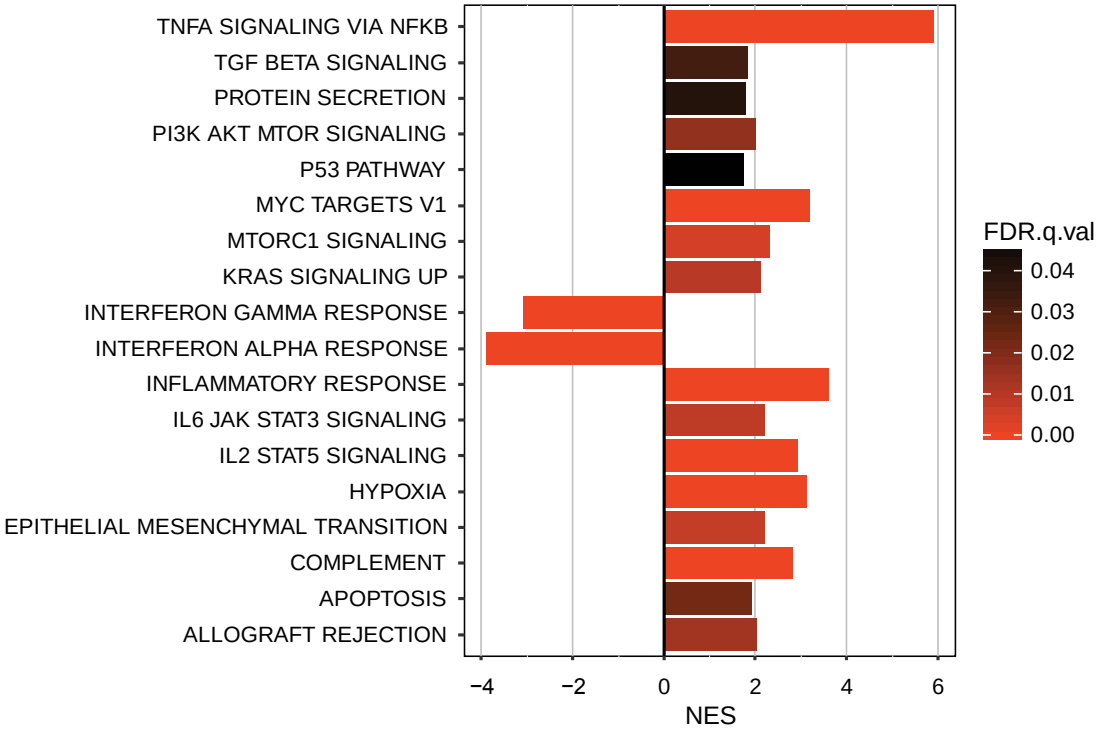

Figure 4.4. Immune complexes induce widespread macrophage activation. GSEA of Hallmarks pathways in macrophages stimulated with O-IC.

However, given their association with UC pathogenesis, we decided to first focus on IC-inducible cytokine/chemokine production (Fig. 4.5A). IC stimulation resulted in increased expression of several key inflammatory cytokines: Th17-inducing *Il1b*, *Il23a*, *Spp1*, and *Il12b*; monocyte and neutrophil-recruiting *Ccl2*, *Ccl7*, *Cxcl1*, and *Cxcl2*; and other UC-associated genes, including *Tnf*, and *Osm*. Interestingly, several anti-inflammatory genes are also induced by O-IC and may act to limit IC-induced inflammation, including *Il10*, as previously mentioned, as well as *Il1rn*, encoding IL-1Ra. Analysis of UC-associated genes induced by IC stimulation revealed a nucleus of Th17-inducing genes associated with both conditions, including *Il1b*, *Il23a*, and *Spp1*, in accordance with previous studies on the pathogenesis of UC and our data suggesting IC can support Th17 immunity (Fig. 4.5B). Notably, UC and IC-stimulated macrophages are both significantly associated with elevated *Cxcl1* and *Cxcl2* expression, implying neutrophil recruitment is a central tenant of both conditions.

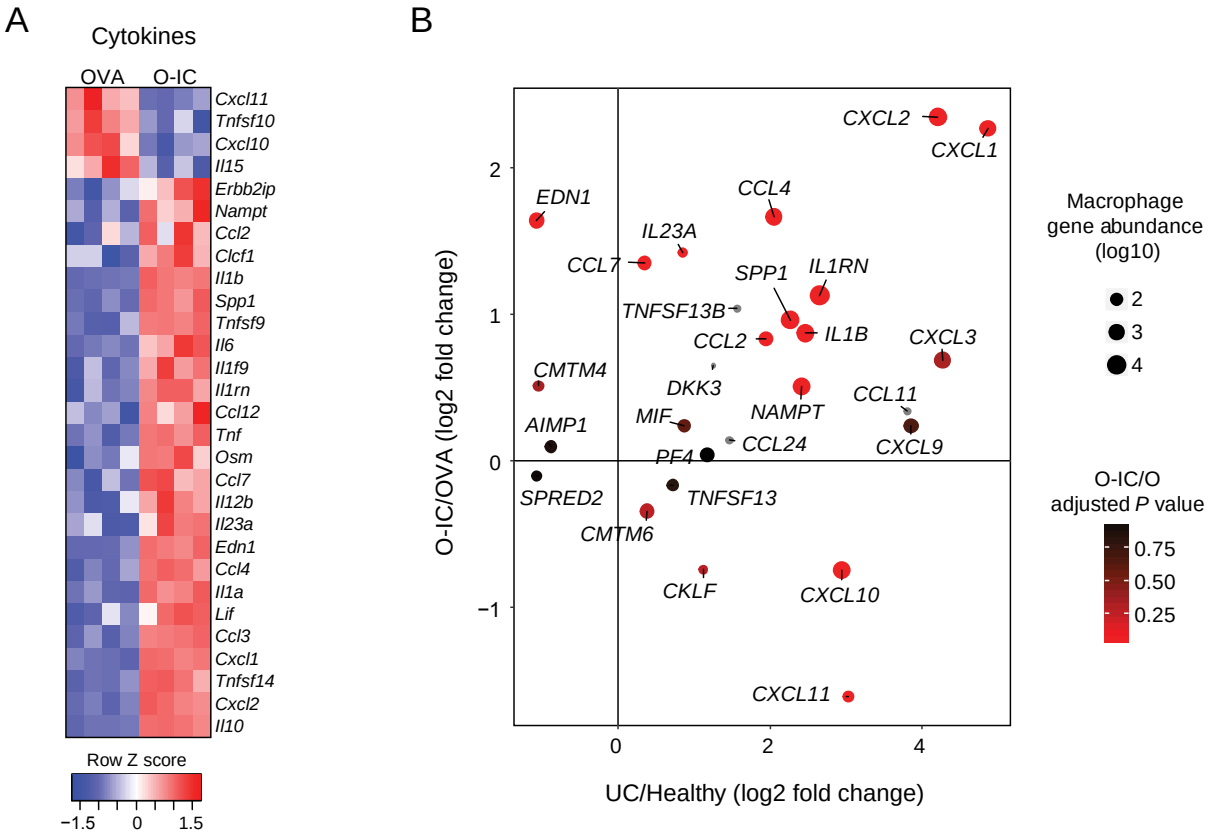

Figure 4.5. Immune complexes induce UC-associated inflammatory gene expression. (A) Heatmap of differentially expressed cytokines and chemokines in intestinal macrophages following IC stimulation. (B) Comparison of intestinal macrophage IC stimulation with significant UC-associated cytokine and chemokine transcripts (adjusted *P* val. < 0.05). *P* values were calculated using the standard DESeq 2 method with multiple correction using BH.

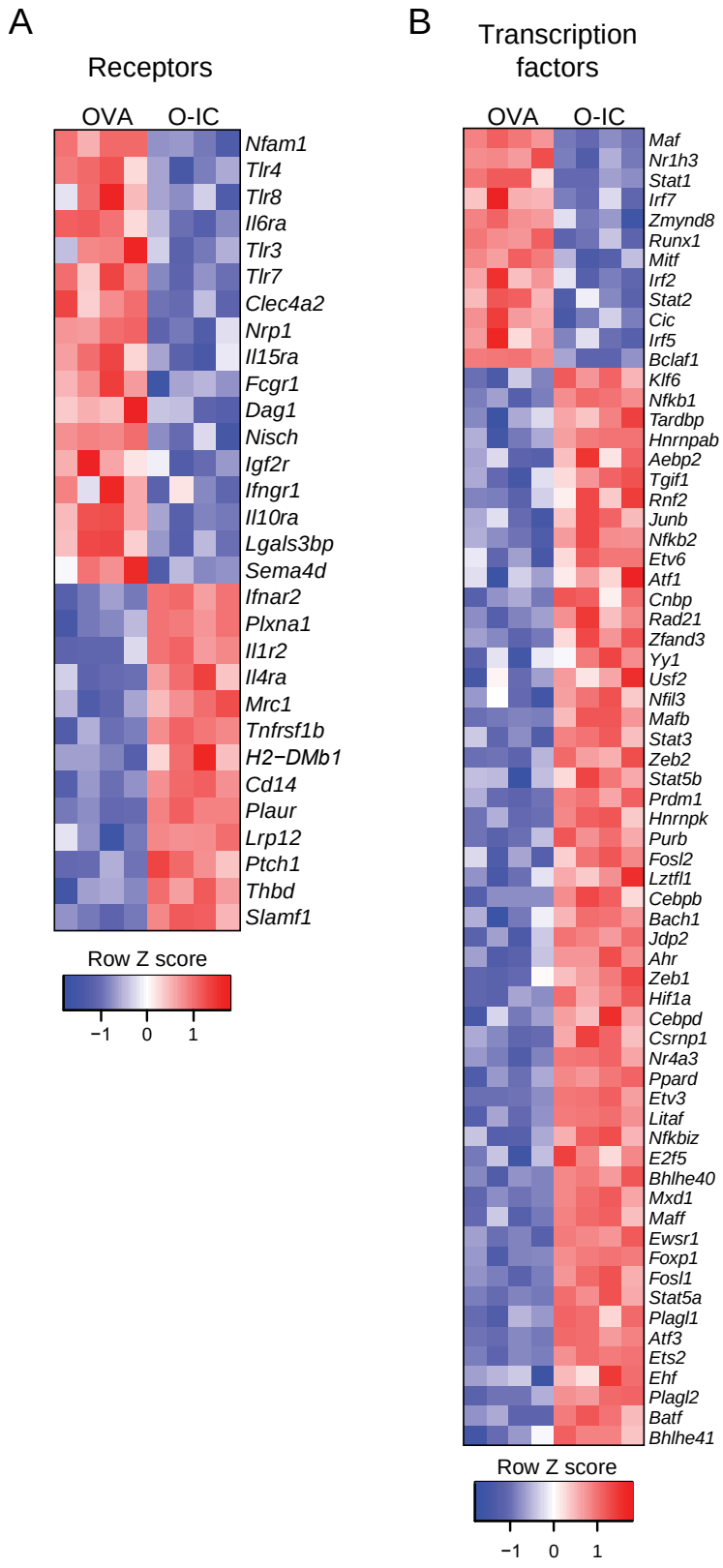

Figure 4.6. Immune complexes regulate macrophage production of cell surface receptors and transcription factors. Heatmaps showing significant differentially expressed cell surface receptors (A) and transcription factor (B) in inflamed flow-sorted intestinal macrophages stimulated with O or O-IC.

Analysis of cell surface receptor and TF expression in intestinal macrophages also demonstrated significant changes in gene expression in response to IC (Fig. 4.6). Several cell surface receptors were downregulated by IC, including *Il10ra* and numerous TLRs (Fig. 4.6A), suggesting a shift in how IC-stimulated cells sense their local environment. Furthermore, there was widespread reprogramming of TFs, with over 50 significantly increased genes following stimulation (Fig. 4.6B), including *Hif1a*, a hypoxia-inducible TF associated with IL-1β production [343], *Stat* genes, and NFκB. One of the most highly induced TRs is *Atf3*, a TF suggested to suppress inflammatory cytokine production, and may represent a negative feedback mechanism to dampen IC-induced responses.

In summary, IC induces widespread inflammatory transcriptional reprogramming of intestinal macrophages centred on the production of Th17-inducing inflammatory cytokines, including IL-1β, and neutrophil-recruiting chemokines, supporting the observed correlations between FcγRs and these same genes in murine and human colitis.

4.3. Manipulation of the A/I ratio using FcγRIIB-transgenic mouse models

FcγR signalling induces a UC-associated inflammatory network in macrophages centred on the production of IL-1β and CXCL1 and CXCL2. Given the importance of these mediators in intestinal inflammation in both mice and humans, we hypothesised that modulation of A/I ratios would alter disease severity via differential production of these cytokines. In humans, this may be through SNPs affecting FcγR expression or function, such as the *FCGR2A* SNP affecting IgG binding affinity, while in mice, this can be artificially manipulated in transgenic mouse models in which FcγR expression is modified.

FcγRIIB is essential for the inhibition of activating FcγR signalling, with *Fcgr2b* KO mice more susceptible to several IgG-mediated inflammatory disorders [62], [63]. As FcγRIIB is most highly expressed on intestinal macrophages, *Fcgr2b* KO mice represent a useful tool to interrogate the function of IgG and FcγR signalling and IgG in intestinal disease progression. In contrast, macrophage transgenic (M-TG) mice overexpress FcγRIIB specifically on macrophages through transgenic *Fcgr2b* expression from the human CD68 promoter [205]. As such, these mice allow for refined cell-specific dissection of the role of FcγR signalling in intestinal disease (Fig. 4.7A).

To determine whether A/I ratio modulation could influence cytokine expression *in vitro*, BMDMs generated from WT, M-TG, and *Fcgr2b*-deficient mice were stimulated with O-IC for 4 h. Cytokine and chemokine production was indeed dependent on the A/I ratio: M-TG BMDMs expressed lower levels of IL-1β, CXCL1 and CXCL2 compared to WT mice in response to O-IC, while *Fcgr2b*-deficient BMDMs demonstrated enhanced cytokine and chemokine production (Fig. 4.7B).

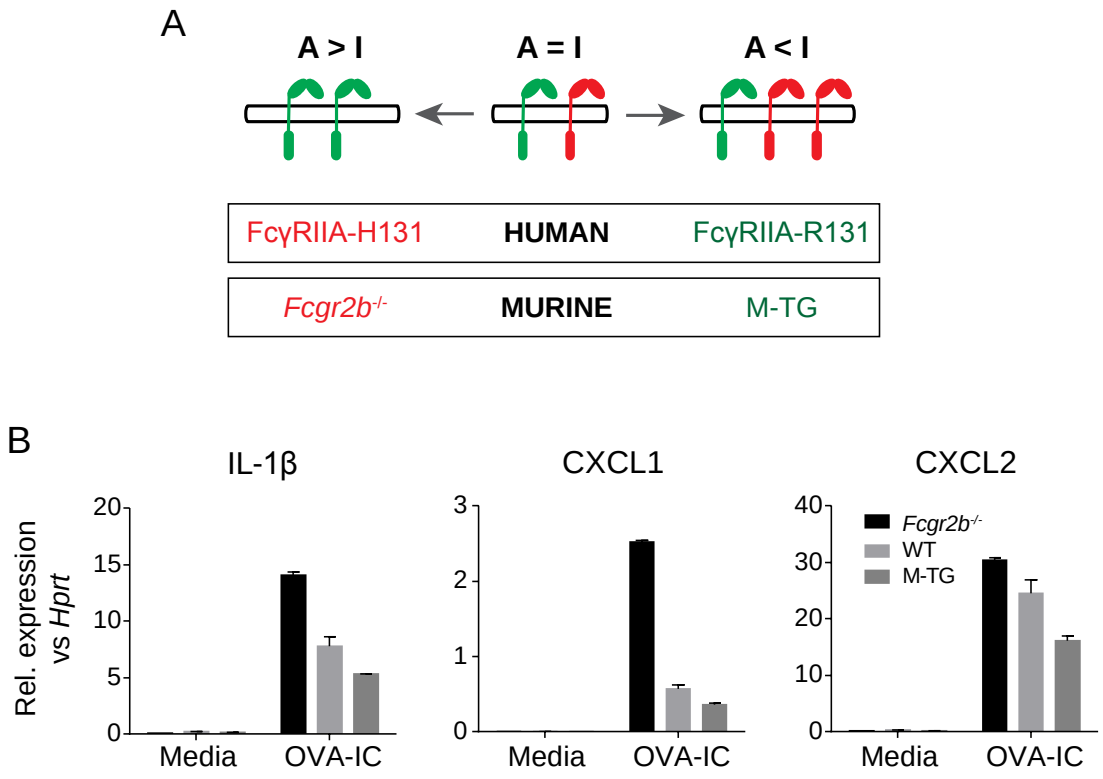

Figure 4.7. Modulation of macrophage A/I ratio regulates inflammatory cytokine production. (A) Schematic of A/I ratio modulation by human *FCGR2A* single nucleotide polymorphisms and transgenic mouse models. (B) qPCR of WT, *Fcgr2b*-deficient, and M-TG BMDMs stimulated with O or O-IC for 3 h. *n* = 2 per group. Data are representative of two independent experiments.

4.4. Chronic DSS-induced colitis in *Fcgr2b*-deficient mice

4.4.1. Clinical parameters

In order to investigate the role of FcγR signalling in chronic intestinal inflammation, *Fcgr2b* KO and WT mice were subjected to chronic DSS administration. DSS administration resulted in weight loss in WT mice, as expected (Fig. 4.8A). *Fcgr2b*-deficient mice exhibited greater weight loss, particularly from day 10 onwards. At endpoint, while no difference was observed in colon length, *Fcgr2b*-deficient colons weighed more and trended to a greater weight per centimetre of tissue (Fig. 4.8B). Furthermore, SLOs were significantly enlarged in *Fcgr2b*-deficient mice following chronic DSS administration (Fig. 4.8C).

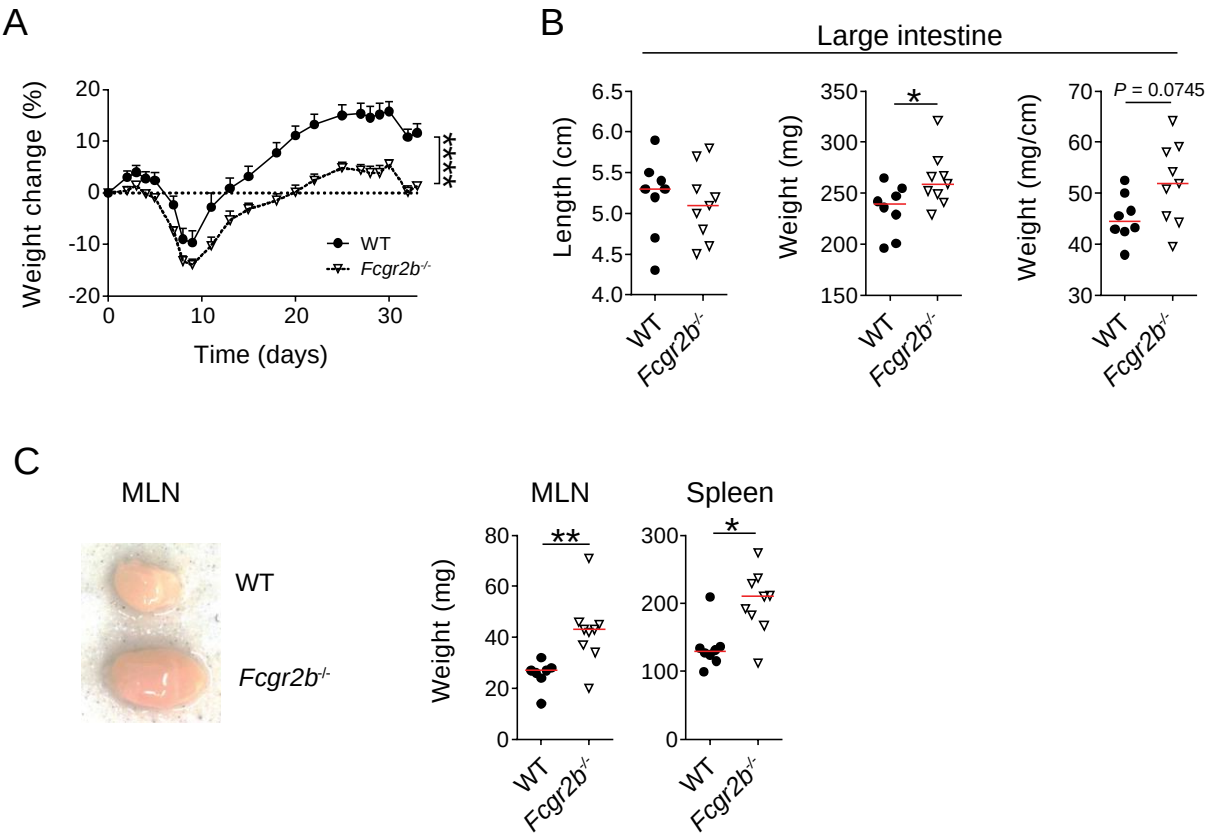

Figure 4.8. Enhanced chronic intestinal disease in *Fcgr2b*-deficient mice. (A) Weight loss in WT and *Fcgr2b*-deficient mice following chronic DSS administration. (B) Colonic clinical disease parameters following cDSS for mice shown in A. (C) MLN and spleen enlargement in WT and *Fcgr2b*-deficient mice following cDSS. Data are representative of three independent experiments. *P* values were calculated using a two-way ANOVA (A), and the nonparametric Mann-Whitney test (B, C). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Confocal microscopy of colonic tissue was performed to investigate leukocyte infiltration and tissue architecture (Fig. 4.9). *Fcgr2b*-deficient mice exhibited increased $CD45^+$ leukocyte infiltration and abscess formation in the mucosa, identified as clusters of $CD45^+$ cells within areas of disrupted mucosal architecture, as well as submucosal expansion, characteristic of enhanced inflammation.

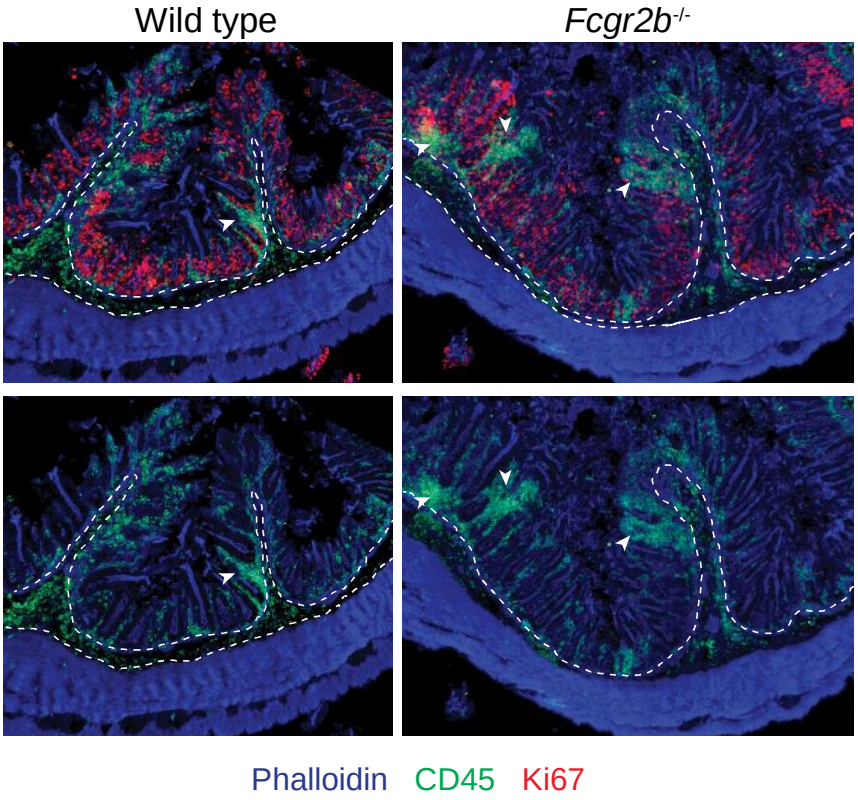

Figure 4.9. Leukocyte infiltration and submucosal expansion in *Fcgr2b*-deficient mice. Confocal microscopy of WT and *Fcgr2b*-deficient colon sections following cDSS. Blue = phalloidin; green = CD45; red = Ki67. Arrows indicate $CD45^+$ leukocyte foci. Data are representative of two independent experiments.

4.4.2. Inflammatory cytokine and chemokine analysis

In order to dissect the mechanistic underpinning of this enhanced disease severity, global cytokine and chemokine expression levels in WT and *Fcgr2b*-deficient whole colon tissue were quantified by qPCR (Fig. 4.10). Notably, of MNP-derived cytokines, global *Il1b* levels were significantly increased (Fig. 4.10A). The genotype effect on *Il23a* and *Tnf* expression was more modest, while minor change was observed in *Tnfsf15* or *Il10* levels. Of the Th17-associated cytokines, an increase in *Il17a* and *Csf2* levels was observed in *Fcgr2b*-deficient colons, while there was also trend towards increasing *Il22* (Fig. 4.10B). Chemokine analysis demonstrated a significant enrichment in *Ccl2* in *Fcgr2b*-deficient colons, while trends towards increasing levels of *Cxcl1* and *Cxcl2* were also seen (Fig. 4.10C). Finally, *Fcgr2b*-deficient colons were associated with an enhanced anti-microbial peptide response, with all *Reg* genes either significantly enriched or trending so compared to WT controls (Fig. 4.10D). Therefore, global cytokine and chemokine expression in *Fcgr2b*-deficient mice is dominated by exacerbated IL-1β production, enhanced Th17 immunity, and increased expression of neutrophil- and monocyte-recruiting chemokines.

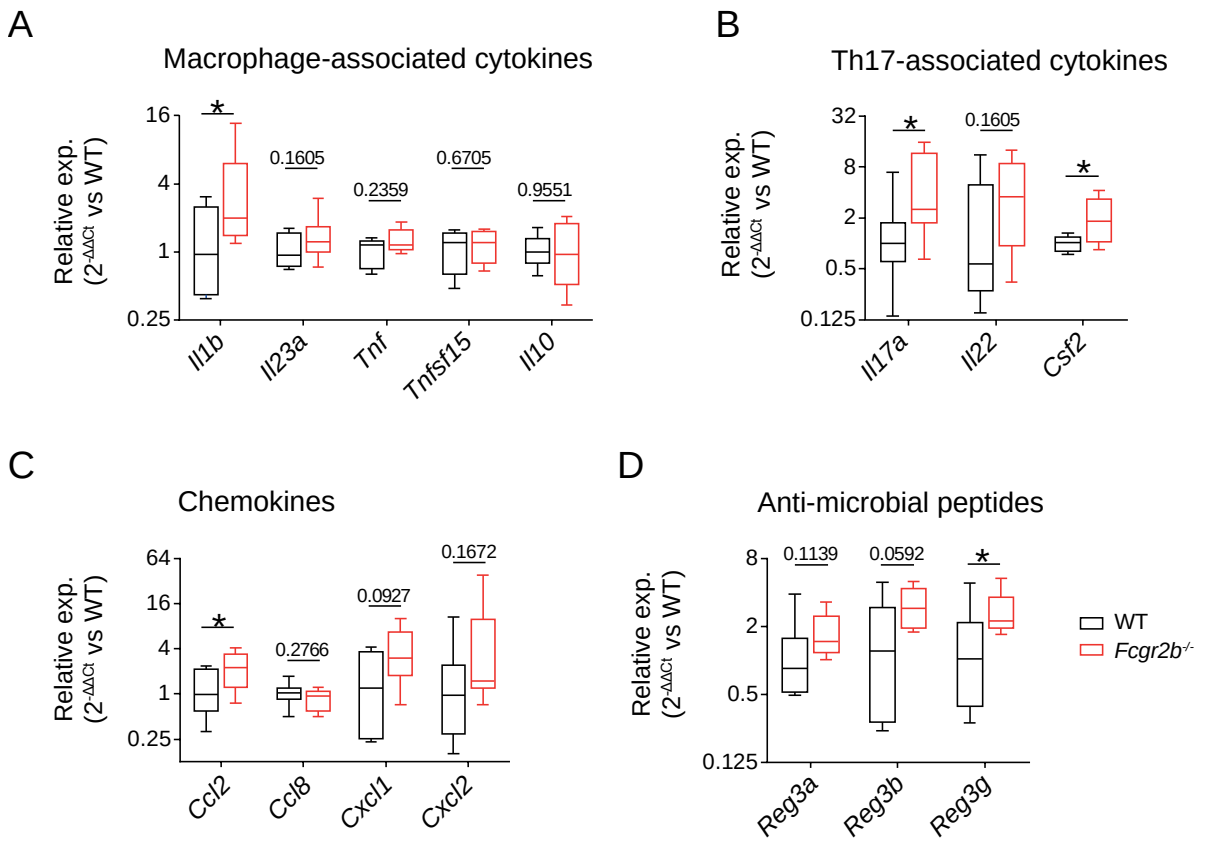

Figure 4.10. FcγRIIB deficiency promotes an inflammatory profile in chronic DSS-induced colitis. qPCR of macrophage-associated cytokines (A), Th17-associated cytokines (B), chemokines (C), and epithelial-derived anti-microbial peptides (D) in whole murine colonic tissue from WT (black) and *Fcgr2b*-deficient (red) mice following cDSS. *n* = 8-9 per group. Data are representative of three independent experiments. *P* values were calculated using the nonparametric Mann-Whitney test. * $P < 0.05$.

The sources of enhanced IL-1β production in *Fcgr2b*-deficient mice were investigated by flow cytometry (Fig. 4.11). Intracellular staining for pro-IL-1β demonstrated that in WT mice, the majority of pro-IL-1β$^+$ cells were CD11b$^+$ CX3CR1$^+$ MNPs, with an equal abundance of Ly6C^{hi} and Ly6C^{lo} cells, consistent with monocytes and macrophages, respectively (Fig. 4.11A). *Fcgr2b*-deficient mice had significantly higher numbers of pro-IL-1β$^+$ cells, the majority of which were Ly6C^{hi} (Fig. 4.11A, B). To address whether this was due to monocytic infiltration or increased IL-1β expression by *Fcgr2b*-deficient monocytes, analysis of the MNP "waterfall" was carried out [68] (Fig. 4.11C). This demonstrated that *Fcgr2b*-deficient monocytes and immature macrophages expressed higher amounts of pro-IL-1β compared to WT cells, with a diminishing genotype effect as monocytes mature into macrophages, consistent with previous observations that newly recruited monocytes are the major source of intestinal IL-1β production. Furthermore, no IL-1β production was observed in splenic monocytes, demonstrating that this a local intestinal effect (Fig. 4.11D).

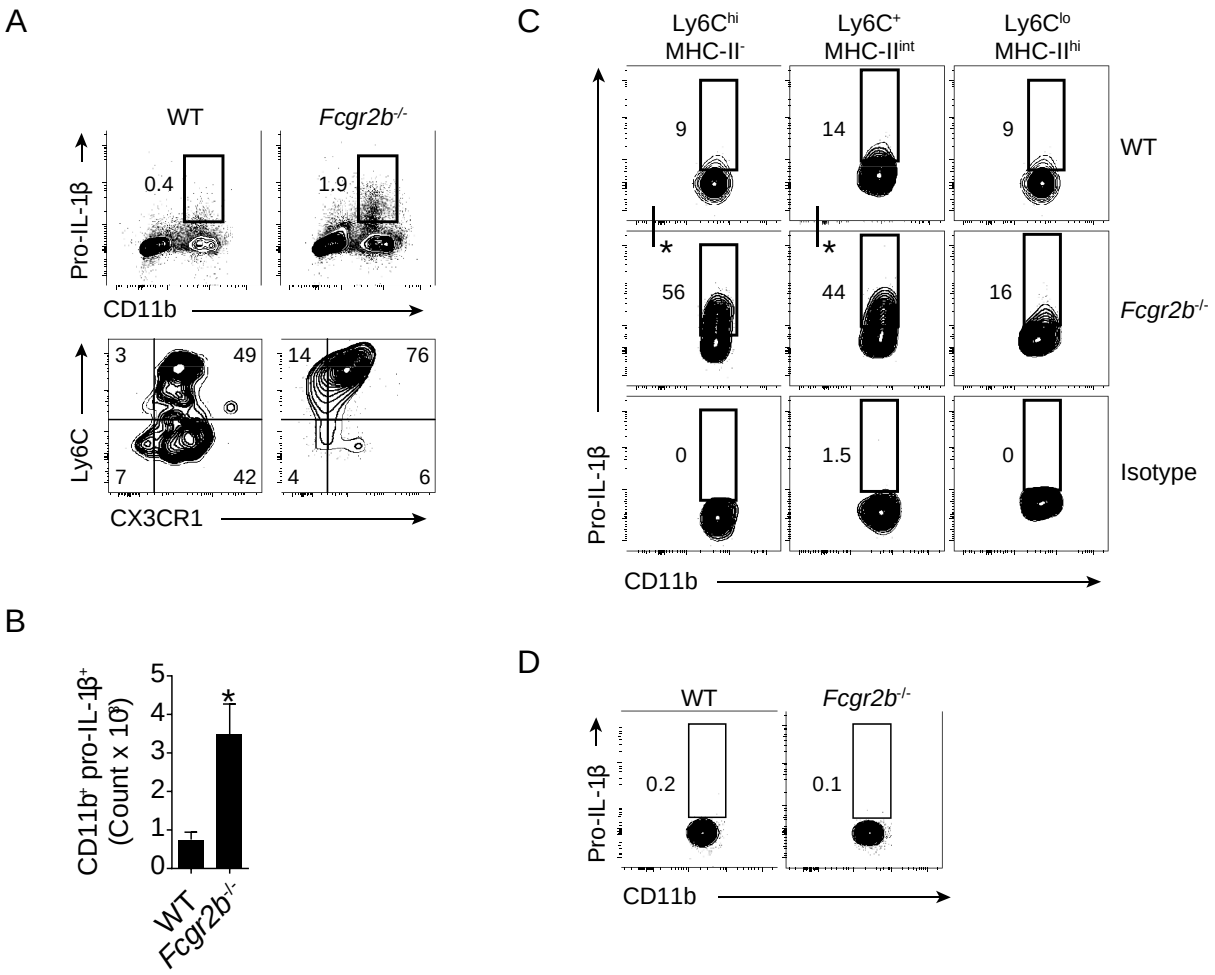

Figure 4.11. Enhanced IL-1β production by intestinal mononuclear phagocytes in the absence of FcγRIIB. (A) Flow cytometry of pro-IL-1β-producing cells in the colonic lamina propria of WT and *Fcgr2b*-deficient mice following cDSS. (B) Quantification of cells in A. (C) Pro-IL-1β production by colonic CX3CR1^{+} MNP subsets following cDSS. (D) Pro-IL-1β production by WT and *Fcgr2b*-deficient splenic monocytes following cDSS. n = 5-7 per group. Data are representative of three independent experiments. P values were calculated with the nonparametric Mann-Whitney test. * $P < 0.05$.

To determine the contribution of dysregulated FcγR signalling on widespread cytokine production by intestinal Ly6C^{hi} MHC-II^{-} monocytes and Ly6C^{lo} MHC-II^{hi} macrophages from the inflamed LI-LP of WT and *Fcgr2b*-deficient mice, flow-sorted cells were analysed by qPCR. In agreement with global mRNA levels, *Il1b* was the most highly expressed cytokine analysed in intestinal macrophages, consistent with a key role in mediating intestinal inflammation (Fig. 4.12A). *Il1b* expression was similar between WT monocytes and macrophages, with its expression significantly enhanced in *Fcgr2b*-deficient monocytes, consistent with protein level data (Fig. 4.12B). *Il1b* expression was also trending to be higher in the macrophage subset. *Il23a* expression followed a similar pattern. As expected, both *Il10* and *Tnf* were most highly expressed in macrophages, with *Tnf* expression significantly higher in *Fcgr2b*-deficient macrophages (Fig. 4.12B). Curiously, no difference in *Il10* expression was seen between WT and *Fcgr2b*-deficient macrophages, but was significantly higher in *Fcgr2b*-deficient monocytes.

Il6 was equally expressed by both monocytes and macrophages, but the effect of FcγRIIB deficiency was most pronounced in the macrophage subset. Finally, no difference in *Tnfsf15* expression was seen in the absence of FcγRIIB, although its expression was much higher in the macrophage subset. These results are largely consistent with inflammatory profiles induced in intestinal macrophages by IC stimulation *ex vivo*, and suggest a prominent role for FcγR signalling on IL-1β production *in vivo*.

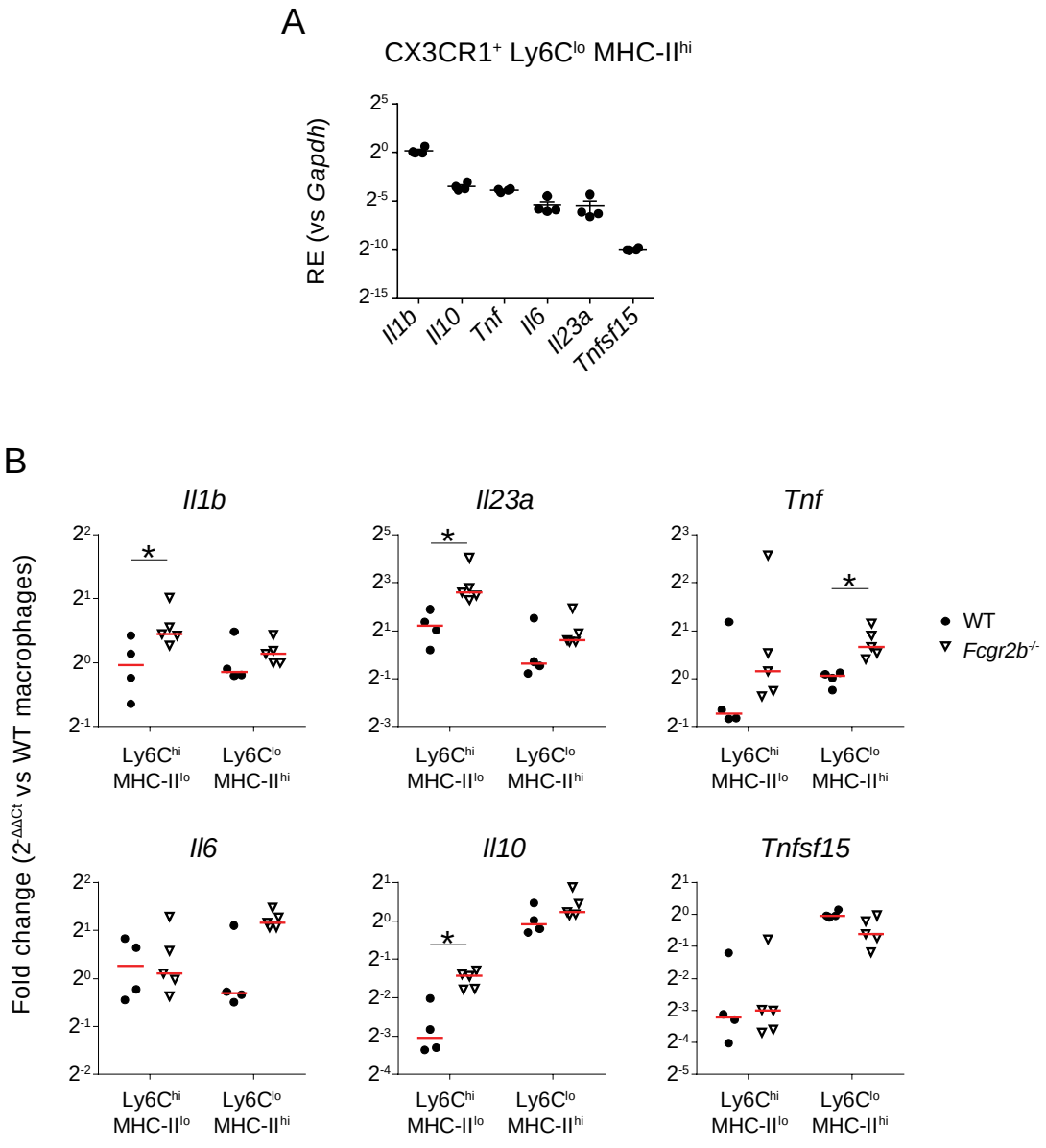

Figure 4.12. Enhanced inflammatory cytokine profile of *Fcgr2b*-deficient CX3CR1^{+} MNPs. (A) qPCR of cytokine gene expression in flow-sorted CX3CR1^{+} Ly6C^{lo} MHC-II^{hi} macrophages 14 days after acute DSS administration, normalised to *Gapdh*. (B) qPCR of inflammatory cytokines in WT and *Fcgr2b*-deficient monocytes (Ly6C^{hi} MHC-II^{lo}) and macrophages (Ly6C^{lo} MHC-II^{hi}) following DSS administration. Data represent a single experiment. *P* values were calculated using the nonparametric Mann-Whitney test. * $P < 0.05$.

Similarly, chemokine expression was analysed in WT and *Fcgr2b*-deficient macrophages by qPCR. *Cxcl2* was the most highly expressed chemokine of those analysed in WT mice (Fig. 4.13A). However, both *Cxcl1* and *Cxcl2* expression were significantly higher in *Fcgr2b*-deficient macrophages, supporting a role for FcγR signalling in neutrophil recruitment via the production of these chemokines (Fig. 4.13B).

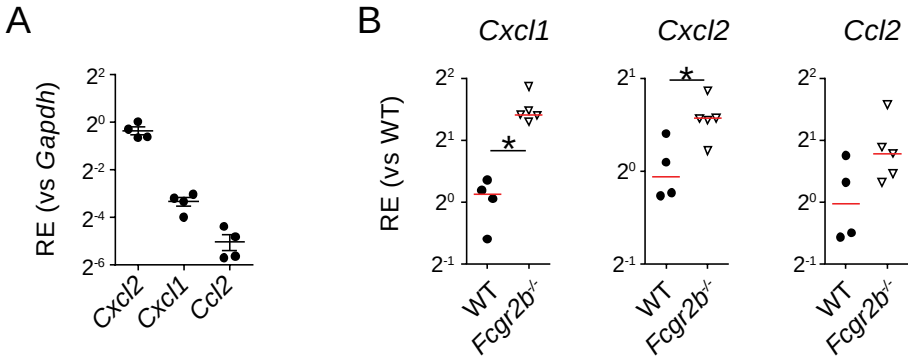

Figure 4.13. Enhanced chemokine production by *Fcgr2b*-deficient CX3CR1^{+} macrophages. (A) qPCR of chemokine expression in WT intestinal macrophages after 14 days of acute DSS, normalised to Gapdh. (B) qPCR of chemokine gene expression by WT and *Fcgr2b*-deficient macrophages following acute DSS administration. Data represent a single experiment. *P* values were calculated using the nonparametric Mann-Whitney test. * $P < 0.05$.

In summary, global FcγRIIB deficiency is associated with enhanced IL-1β production by intestinal CX3CR1^{+} MNPs, with a particular effect on newly recruited Ly6C^{hi} monocytes and immature Ly6C^{+} MHC-II^{+} macrophages. Furthermore, IL-1β was the most highly expressed of all inflammatory cytokines investigated, and likely represents a dominant effect of the genotype, as expression level differences for other cytokines were less apparent in *Fcgr2b*-deficient mice versus controls.

4.4.3. Leukocyte profiling of the inflamed colon

Given the increased expression of several monocyte- and neutrophil-recruiting chemokines in *Fcgr2b*-deficient mice, we sought to investigate myeloid cell infiltration into the inflamed colon of these mice. Confocal microscopy demonstrated a pronounced enrichment of CD11b^{+} cells within the inflamed submucosa of *Fcgr2b*-deficient mice compared to WT controls following chronic DSS administration (Fig. 4.14).

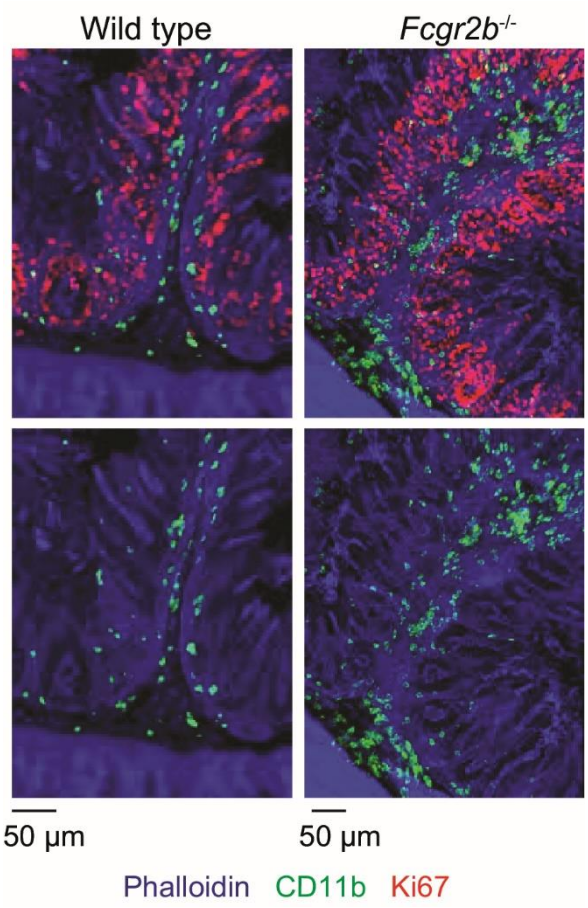

Figure 4.14. Submucosal CD11b$^+$ leukocyte infiltration in *Fcgr2b*-deficient mice. Confocal microscopy of WT and *Fcgr2b*-deficient colonic tissue following cDSS. Blue = phalloidin; green = CD11b; red = Ki67. Data are representative of two independent experiments.

Flow cytometric tSNE analysis of chronically inflamed colons demonstrated enrichment of colonic neutrophils in *Fcgr2b*-deficient mice (Fig. 4.15A). This was reflected in an increase in the absolute number of these cells, as well as SSC^{hi} CD11b$^+$ granulocytes (Fig. 4.15B). An enrichment of monocytes was also observed in the absence of FcγRIIB (Fig. 4.15A). Analysis of the CX3CR1$^+$ MNP waterfall demonstrated an enrichment of mucosal monocyte frequency and absolute number in the absence of FcγRIIB (Fig. 4.15C). No change was detected in systemic monocyte levels, consistent with a local effect on monocyte recruitment (Fig. 4.15D). However, systemic neutrophil levels were increased in the absence of FcγRIIB, consistent with increased bone marrow neutrophil mobilisation (Fig. 4.15D).

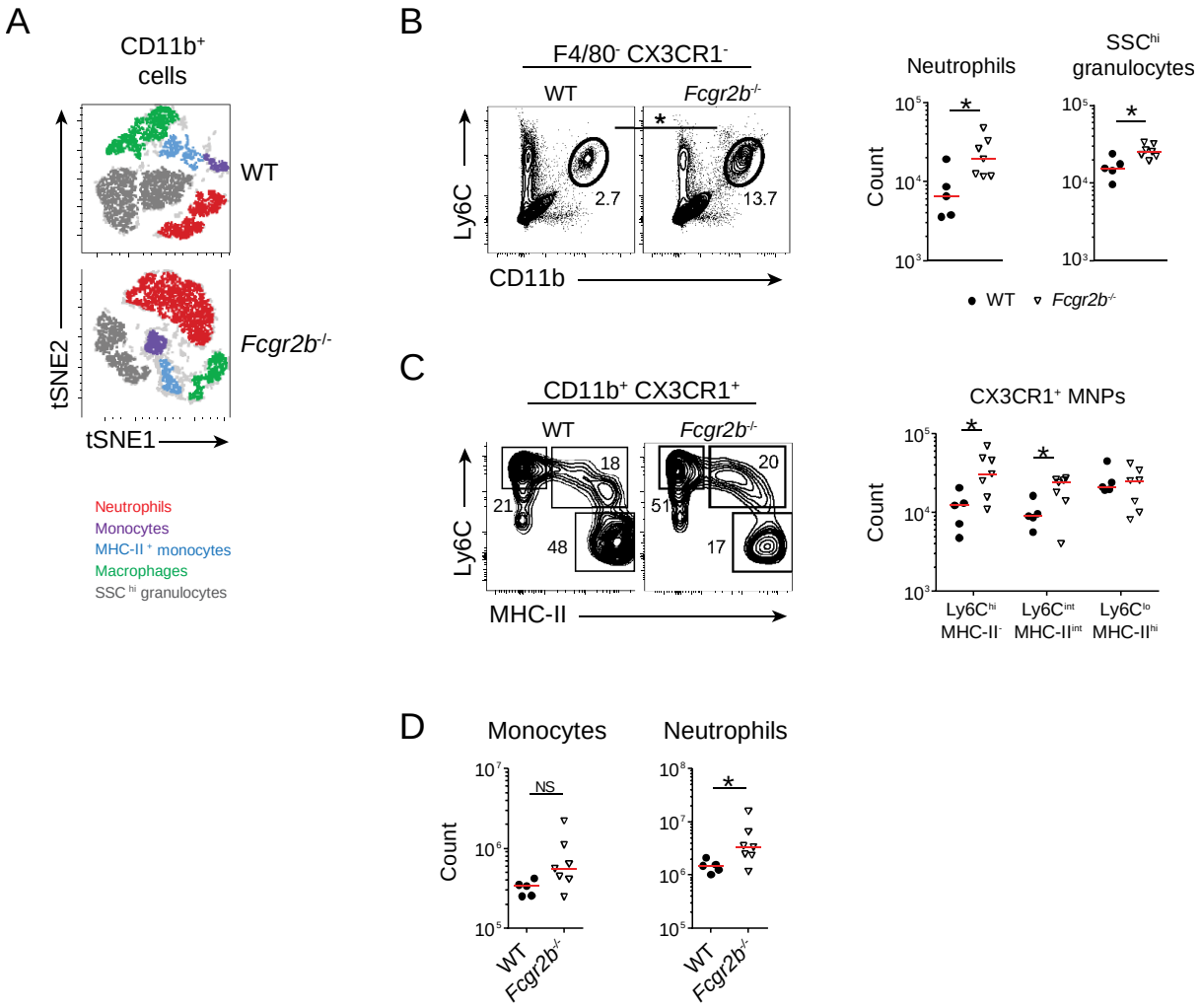

Figure 4.15. Intestinal neutrophil and monocyte infiltration in the absence of FcγRIIB. (A) tNSE analysis of colonic CD11b$^+$ cells by flow cytometry. (B) Flow cytometry showing colonic neutrophil infiltration in WT and *Fcgr2b*-deficient mice following cDSS, and quantification of colonic neutrophil and granulocyte numbers. (C) Flow cytometry gating and quantification of CX3CR1$^+$ MNP subsets following cDSS. (D) Splenic monocyte and neutrophil counts following cDSS. Data are representative of three independent experiments. *P* values were calculated using the nonparametric Mann-Whitney test. * $P < 0.05$.

Finally, given the enlargement of SLOs in *Fcgr2b*-deficient mice following DSS-induced colitis, and the role of FcγRIIB in regulating B cell activation and survival, we sought to profile the B cell phenotype in mice following chronic intestinal inflammation. Activated B cells were enriched in *Fcgr2b*-deficient mice. Antibody isotype staining demonstrated an increased proportion and absolute number of class-switched IgD$^-$ IgM$^-$ B cells in both MLNs and spleens of *Fcgr2b*-defcient mice (Fig. 4.16A, B). GL7 staining is a marker of GC activation. The number and proportion of GL7$^+$ IgD$^-$ B cells was similarly increased in SLOs of *Fcgr2b*-deficient mice (Fig. 4.16C, D).

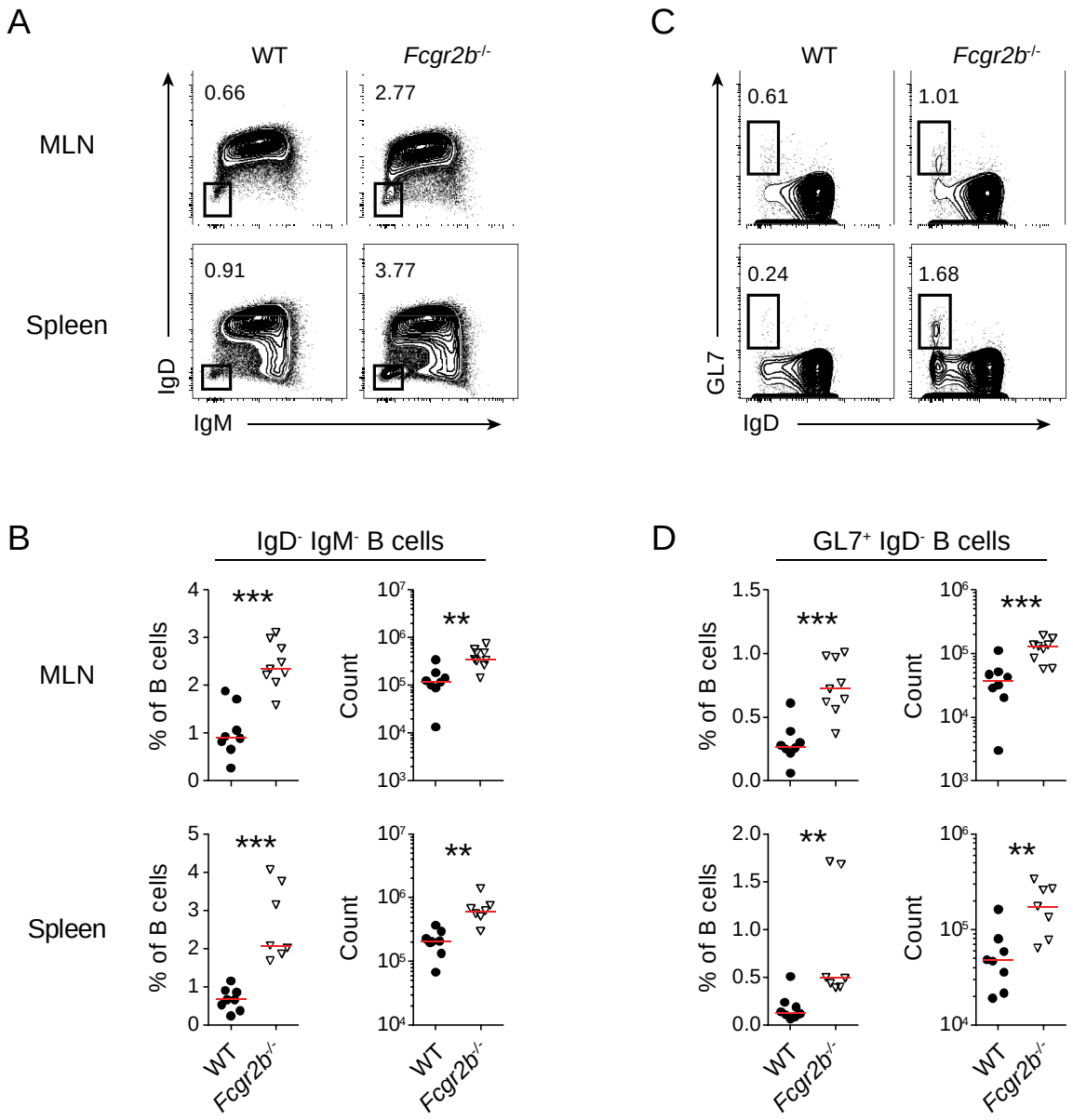

Figure 4.16. Dysregulated B cell activation in secondary lymphoid organs in *Fcgr2b*-deficient mice. (A) Flow cytometry of MLN and splenic CD19$^+$ B220$^+$ class-switched B cells following cDSS. (B) Quantification of cells shown in A. (C) Flow cytometry of MLN and splenic germinal centre CD19$^+$ B220$^+$ B cells following cDSS. (D) Quantification of cells in C. Data represent a single experiment. *P* values were calculated using the nonparametric Mann-Whitney test. ** $P < 0.01$; *** $P < 0.001$.

In summary, *Fcgr2b*-deficient mice exhibit a more severe chronic intestinal disease course, characterised by exacerbated tissue inflammation, enhanced local MNP IL-1β and chemokine production, colonic monocyte and granulocyte infiltration, and a hyperactivated B cell phenotype in local and systemic SLOs. These results serve to demonstrate the importance of the previously identified inflammatory networks in the development and progression of intestinal inflammation *in vivo*.

4.4.4. IL-1β blockade in *Fcgr2b*-deficient mice

IL-1β has well established roles in the activation and maintenance of IL-17A-producing T cell subsets *in vitro* and *in vivo,* while IL-1β neutralisation can suppress DSS- and *H. hepaticus*-induced inflammation. We therefore hypothesised that exacerbated mucosal immune responses in *Fcgr2b*-deficient mice may be abrogated by blockade of IL-1R1 signalling. To achieve this, WT and *Fcgr2b*-deficient mice were treated with anti-IL-1R1 blocking IgG antibody or an equivalent amount of Syrian hamster IgG in serum at weekly intervals during a course of DSS administration.

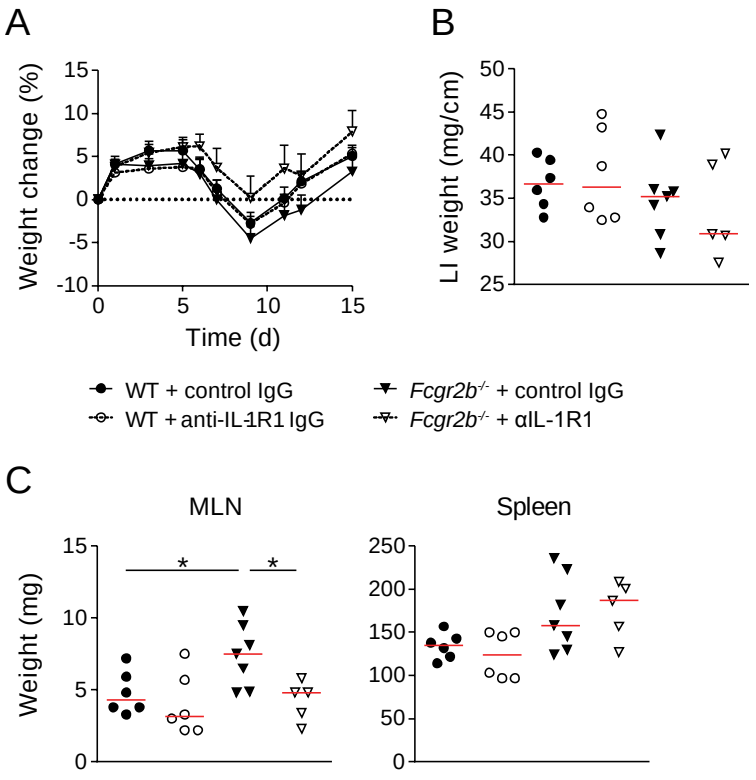

Figure 4.17. IL-1R1 blockade improves clinical disease severity in *Fcgr2b*-deficient mice. (A) Weight change following acute DSS administration and treatment with control or anti-IL-1R1 IgG antibody. (B, C) Colon weight (B) and SLO weight (C) at day 15 post-DSS administration of mice shown in A. Data are representative of two independent experiments. *P* values were calculated using the nonparametric Mann-Whitney test. * $P < 0.05$.

Fcgr2b-deficient mice lost more weight than WT mice on DSS, although this was not as significant as previous experiments, while those receiving anti-IL-1R1 IgG lost less weight compared to littermates receiving control IgG (Fig. 4.17A). While not significant, this may be limited by the relatively small number of mice per group (5-6). Analysis of colon weight demonstrated a trend towards reduced colon weight per centimetre in *Fcgr2b*-deficient mice treated with anti-IL-1R1 IgG compared to control mice (Fig. 4.17B). Interestingly, while splenic weight remained unchanged with anti-IL-1R1 treatment, MLN weight was significantly reduced

in *Fcgr2b*-deficient mice receiving anti-IL-1R1 IgG, supportive of reduced local inflammation (Fig. 4.17C). Of note, there was less pronounced clinical differences between WT and *Fcgr2b*-deficient mice compared to previous experiments. This may be due to the duration of disease course, as samples were collected at day 15, rather than the later timepoints used in previous experiments. Furthermore, colitis induction was not as successful in this case, as demonstrated by relatively minor changes in weight loss despite 7 days of DSS administration (Fig. 4.17A). Strikingly, treatment of WT mice with anti-IL-1R1 IgG did not significantly affect any measure of clinical disease severity.

Flow cytometric analysis of the colon demonstrated that while *Fcgr2b*-deficient mice exhibited enhanced colonic neutrophil recruitment relative to WT controls, IL-1R1 blockade restored neutrophil numbers back to WT levels in *Fcgr2b*-deficient mice (Fig. 4.18A). No change in neutrophils was seen in WT mice treated with anti-IL-1R1 IgG. Systemic neutrophils, as demonstrated by staining in the spleen, were also trending towards a reduction in frequency and absolute number following IL-1R1 blockade (Fig. 4.18B).

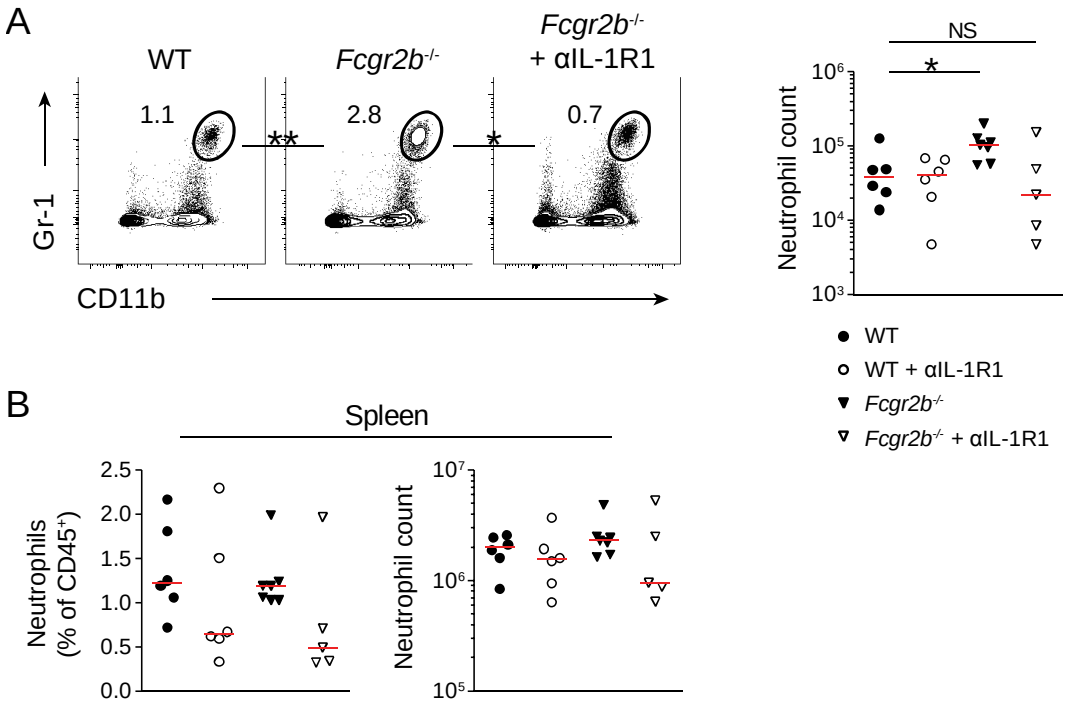

Figure 4.18. Reduced colonic neutrophil infiltration following IL-1R1 blockade. (A) Representative flow cytometry plots showing $CD11b^+$ $Ly6C/G^+$ neutrophils in the colonic LP at day 15 post-DSS administration and treatment with antibodies. (B) Analysis of splenic neutrophil populations at day 15 post-DSS. Data are representative of two independent experiments. *P* values were calculated using the nonparametric Mann-Whitney test. * $P < 0.05$; ** $P < 0.01$.

In contrast to the effect on neutrophils, anti-IL-1R1 IgG administration had a smaller effect on CX3CR1^{+} MNP numbers in colon of *Fcgr2b*-deficient mice (Fig. 4.19A). While colonic CX3CR1^{+} MNP subsets were once again increased in frequency and number in *Fcgr2b*-deficient mice relative to WT controls, IL-1R1 blockade in these mice, or WT mice, had little effect (Fig. 4.19B). This supports a role for IL-1β downstream of monocyte recruitment. Furthermore, IL-1R1 blockade had a negligible effect on splenic monocyte numbers (Fig. 4.19C).

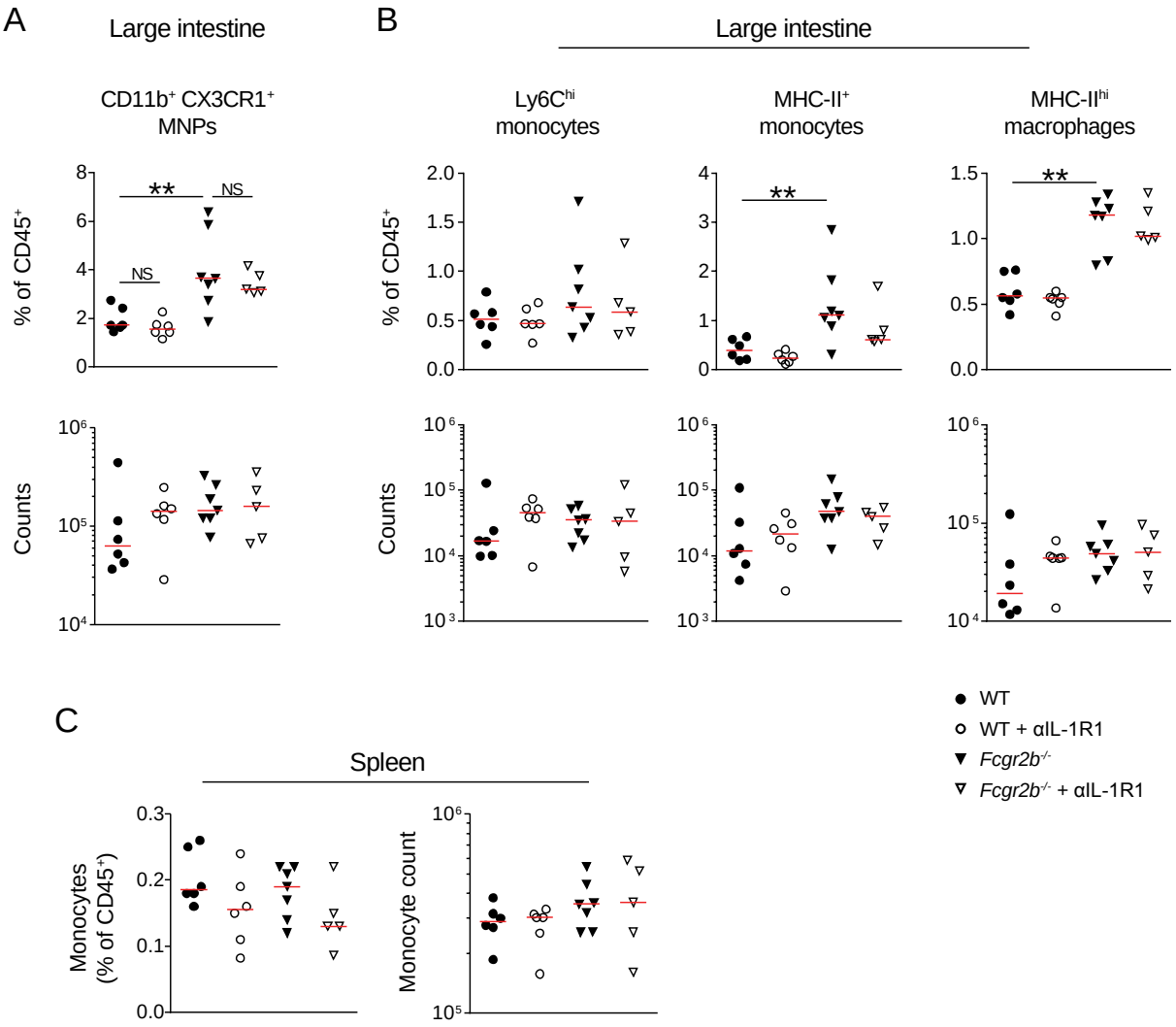

Figure 4.19. IL-1R1 blockade does not affect frequency or number of MNP subsets. (A) Flow cytometric quantification of total CX3CR1^{+} cells in the colonic lamina propria. (B) Quantification of CX3CR1^{+} MNP subsets by flow cytometry. (C) Quantification of splenic monocyte frequency and number. All samples are taken at day 15 post-DSS administration. Data are representative of two independent experiments. *P* values were calculated using the nonparametric Mann-Whitney test. ** $P < 0.01$.

IL-1R1 blockade did not profoundly affect IL-1β production by CD11b$^+$ CX3CR1$^+$ MNP subsets, as demonstrated by flow cytometry, in both WT and *Fcgr2b*-deficient mice (Fig. 4.20). However, the variability of IL-1β staining in CX3CR1$^+$ MNP subsets in this case does not allow for complete dissection of the effect of IL-1R1 blockade on this aspect of MNP biology.

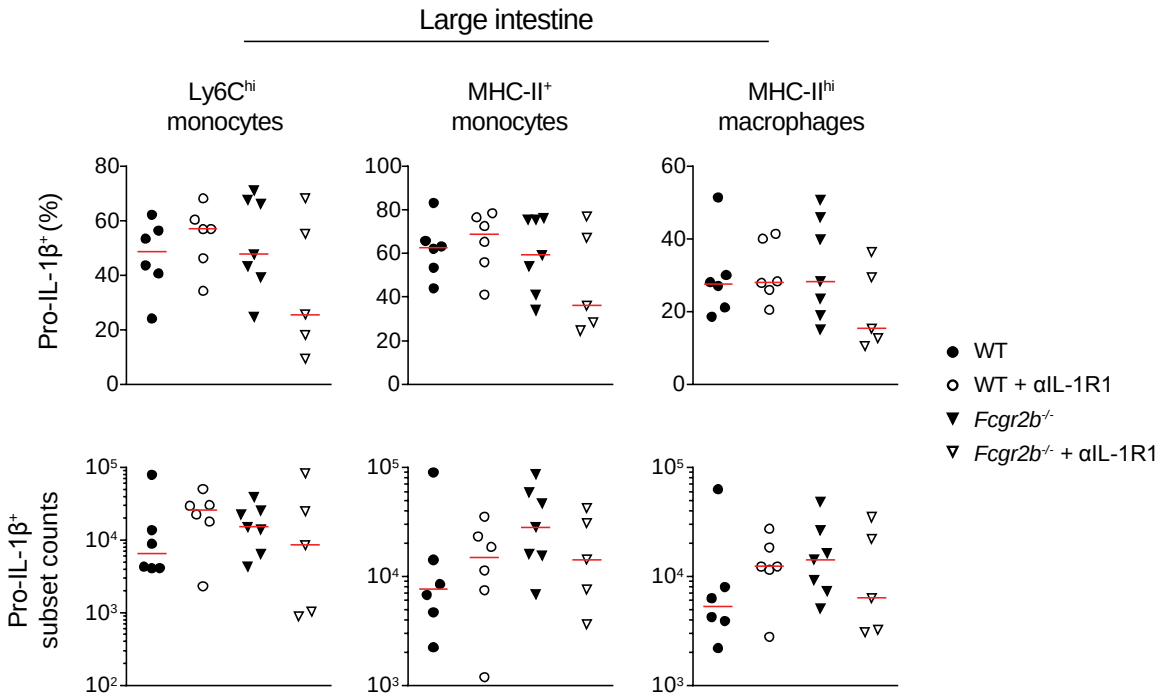

Figure 4.20. IL-1R1 blockade slightly reduces colonic MNP IL-1β production. Quantification of percentage and absolute number of colonic pro-IL-1β$^+$ MNP subsets as determined by flow cytometry at day 15 post-DSS administration. Data are representative of two independent experiments.

4.4.5. Analysis of intestinal Th17 immunity in *Fcgr2b*-deficient mice

We hypothesised that *Fcgr2b*-deficient mice would exhibit enhanced colonic IL-1β-dependent Th17 numbers, given the increase of global IL-1β and IL-17A, IL-22, and GM-CSF levels in *Fcgr2b*-deficient mice (Fig. 4.10).

Colonic IL-17A-producing T cells were significantly increased in *Fcgr2b*-deficient mice following chronic DSS administration, as determined by flow cytometry (Fig. 4.21A). Total CD3ε$^+$ T cell numbers were increased in the absence of FcγRIIB (Fig. 4.21B), with an increased frequency of T cells expressing IL-17A$^+$, as well as an increase in the total number of IL-17A$^+$ T cells (Fig. 4.21B). While the total number of T cells was unaffected by treatment, the increase in IL-17A production was IL-1β-dependent, as administration of the anti-IL-1R1 blocking antibody reduced IL-17A-producing T cells back to near WT levels. T cells were also subdivided into γδ CD4$^-$ T cells (Fig. 4.21C) and CD4$^+$ αβ T cells (Fig. 4.21D), with increases in the total number of both subsets in the inflamed colons of *Fcgr2b*-deficient mice that was IL-1β-independent. Once more, there was an increase in the frequency of T cells expressing IL-

17A and an increase in total number of IL-17A-producing T cells in the absence of *Fcgr2b*, with a larger effect within the γδ T cell pool. These effects were IL-1β-dependent, with a significant reduction in frequency and absolute numbers of IL-17A-producing cells in the presence of anti-IL-1R1 blocking antibody. Finally, while IL-1R1 blockade trended towards reducing the frequency of WT IL-17A-producing T cells, this was not translated into a reduction in absolute numbers of IL-17A-producing cells. Therefore, these effects were specific to T cells in *Fcgr2b*-deficient mice.

IL-22 production by intestinal T cells was also investigated. While IL-22-producing γδ T cells and $CD4^+$ αβ T cells were not as prevalent as their IL-17A-producing counterparts, there remained a significant increase in IL-22 production in the absence of FcγRIIB in both subsets (Fig. 4.22). This was apparent as an increase in the frequency of T cells that expressed IL-22 and the total number of IL-22-producing T cell subsets. There was a trend towards the reduction in the frequency of IL-22 producing T cells following IL-1R1 blockade in both subsets in *Fcgr2b*-deficient mice, but not WT mice, as well as a significant reduction in the total number of $IL\text{-}22^+$ $CD4^+$ αβ T cells in the absence of IL-1R1 signalling. ILC3s are major sources of intestinal IL-22 and are known to be regulated by IL-1 signalling. This will be discussed in Chapter 5.

MLNs drain the intestinal mucosa and are the major site of local T cell activation. We identified the same cell T cell subsets in the MLN, and once again demonstrated an increase in both γδ T cells and $CD4^+$ αβ T cells locally that was unaffected by IL-1β blockade. However, little IL-17A production was observed compared to the colon, and this was largely unaffected by anti-IL-1R1 IgG administration. This is consistent with the role of IL-1β as an inflammatory cytokine that sustains local IL-17A production within the mucosa, rather than influencing Th17 cell generation in draining lymph nodes. Curiously, the enrichment of MLN $CD19^+$ B cells seen in *Fcgr2b*-deficient mice was, at least in part, dependent on IL-1 signalling.

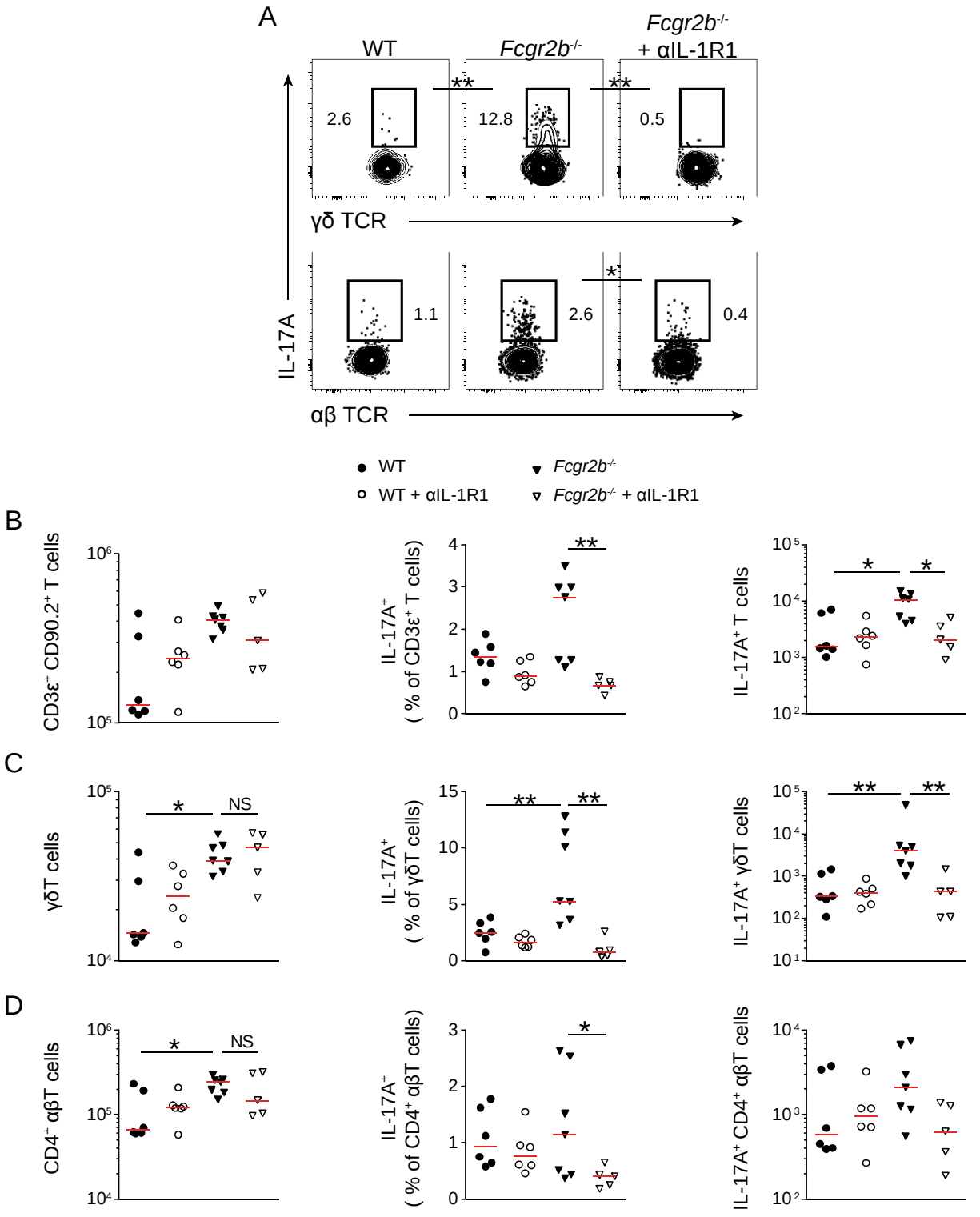

Figure 4.21. Enhanced intestinal IL-1β-dependent Th17 cells in *Fcgr2b*-deficient mice. (A) Flow cytometry of IL-17A production by intestinal T cell subsets in WT or *Fcgr2b*-deficient mice treated with a blocking anti-IL-1R1 or control antibody, following 15 days post-acute DSS administration. (B-D) Quantification of total (left) and IL-17A-producing (middle, right) colonic CD3ϵ+ T cells (B), γδ T cells (C), and αβ T cells (D). Data are representative of two independent experiments. *P* values were calculated using the nonparametric Mann-Whitney test. * $P < 0.05$; ** $P < 0.01$.

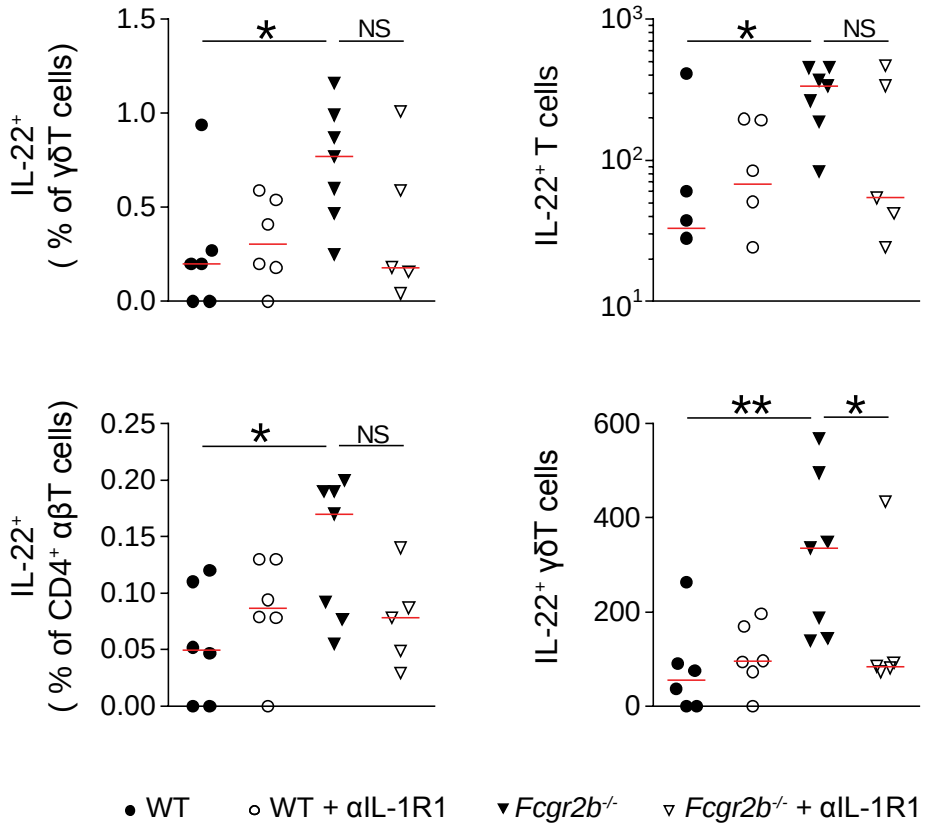

Figure 4.22. IL-22-producing T cells are increased in *Fcgr2b*-deficient mice. Quantification of the frequency (left) and absolute number (right) of colonic IL-22-producing γδ T cells (top row) and αβ T cells (bottom row) in WT and *Fcgr2b*-deficient mice following cDSS. Data are representative of two independent experiments. *P* values were calculated using the nonparametric Mann-Whitney test. * $P < 0.05$; ** $P < 0.01$.

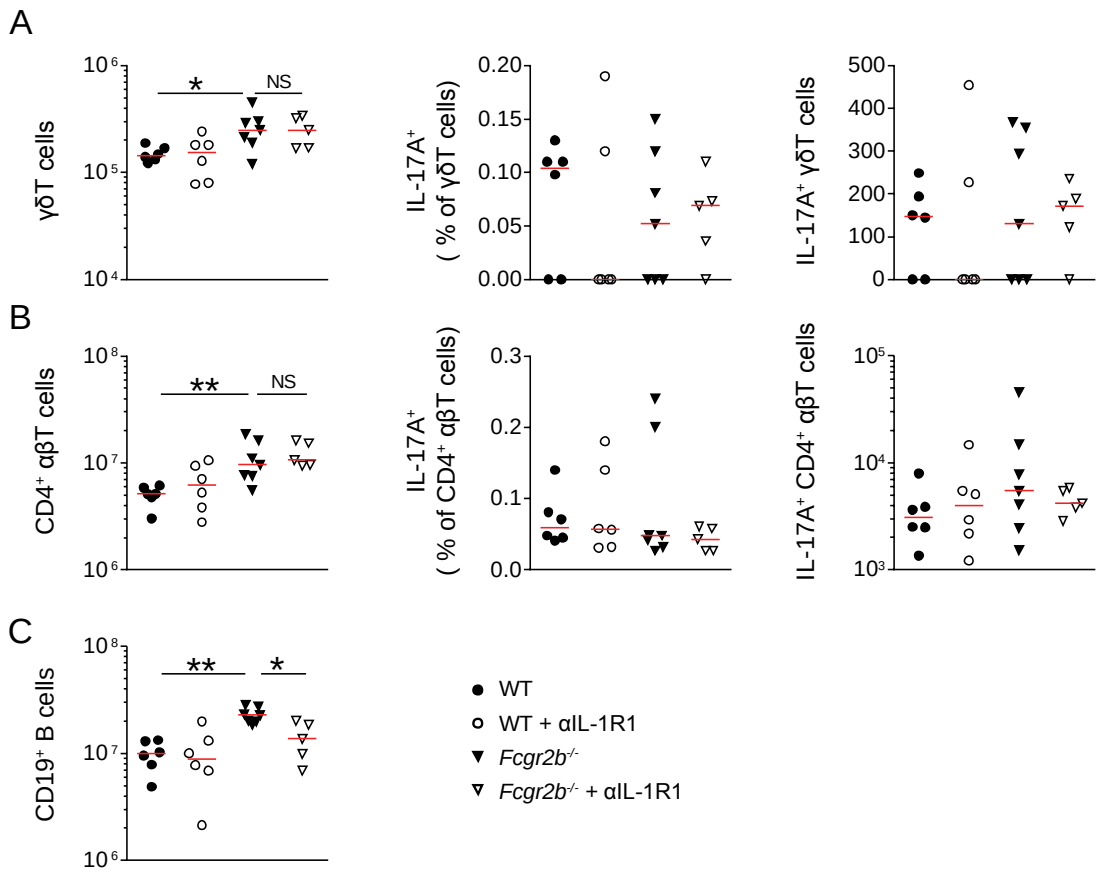

Figure 4.23. The effect of IL-1R1 blockade on MLN-resident lymphocyte populations. (A, B) Flow cytometric quantification of the total number (left), percentage IL-17A$^+$ (middle), and absolute number of IL-17A$^+$ (right) γδ T cells (A) and CD4$^+$ αβ T cells (B) in MLNs at day 15 post-DSS administration in WT or *Fcgr2b*-deficient mice treated with anti-IL-1R1 blocking antibody or control IgG. (C) Quantification of total CD19$^+$ B cells in the MLN by flow cytometry. Data are representative of two independent experiments. *P* values were calculated using the nonparametric Mann-Whitney test. * $P < 0.05$; ** $P < 0.01$.

In summary, while the earlier endpoint for this experiment (day 15) may have diminished the chronic effect of FcγRIIB deficiency on certain features of inflammation, cellular differences between WT and *Fcgr2b*-deficient mice remained apparent. Mice lacking inhibitory FcγRIIB exhibit enhanced IL-17A and IL-22 production by CD3ε$^+$ T cells, in particular γδ T cells, within the inflamed colonic mucosa, while also exhibiting an increase in the total numbers of T cell subsets residing within the inflamed tissue. Furthermore, anti-IL-1R1 blockade dampened exacerbated immunity in *Fcgr2b*-deficient mice, with a restoration of neutrophil recruitment and IL-17A and IL-22 production by T cells back to WT levels. Strikingly, however, several other features of FcγRIIB deficiency remain unaccounted for by IL-1β blockade, including monocyte recruitment, cytokine production by CD11b$^+$ CX3CR1$^+$ MNP subsets, and T cell expansion, consistent with a role of FcγR signalling on a wide range of immune mechanisms. Importantly, anti-IL-1R1 IgG treatment is only effective in *Fcgr2b*-deficient mice.

4.5. Chronic DSS-induced colitis in macrophage transgenic mice

4.5.1. Clinical parameters

Although FcγRIIB expression is highest among CX3CR1^{+} MNPs within the GI tract, the widespread expression profile of this receptor means that certain clinical manifestations in *Fcgr2b*-deficient mice may result from broad immune hyperactivity. As such, refined models of FcγR signalling are required to assess the cell-intrinsic role of FcγR signalling *in vivo* on cell types of interest. To investigate the cell intrinsic role of FcγRIIB on macrophages *in vivo*, macrophage M-TG mice were used. Firstly, macrophage-specific overexpression of FcγRIIB was confirmed within the intestinal MNP populations by flow-cytometry (Fig. 4.24). Differential FcγRIIB expression was most prominent in Ly6C^{hi} MHC-II^{lo} monocytes and immature macrophages (Fig. 4.24A). Consequently, A/I ratios for all activating receptors were significantly reduced in M-TG mice, making this model useful for discriminating the cell-intrinsic effect of FcγRIIB in intestinal MNPs (Fig. 4.24B). FcγRIIB overexpression did not influence expression of other FcγRs on intestinal MNPs (Fig. 4.24C) and was highly specific to the macrophage lineage (Fig. 4.24D).

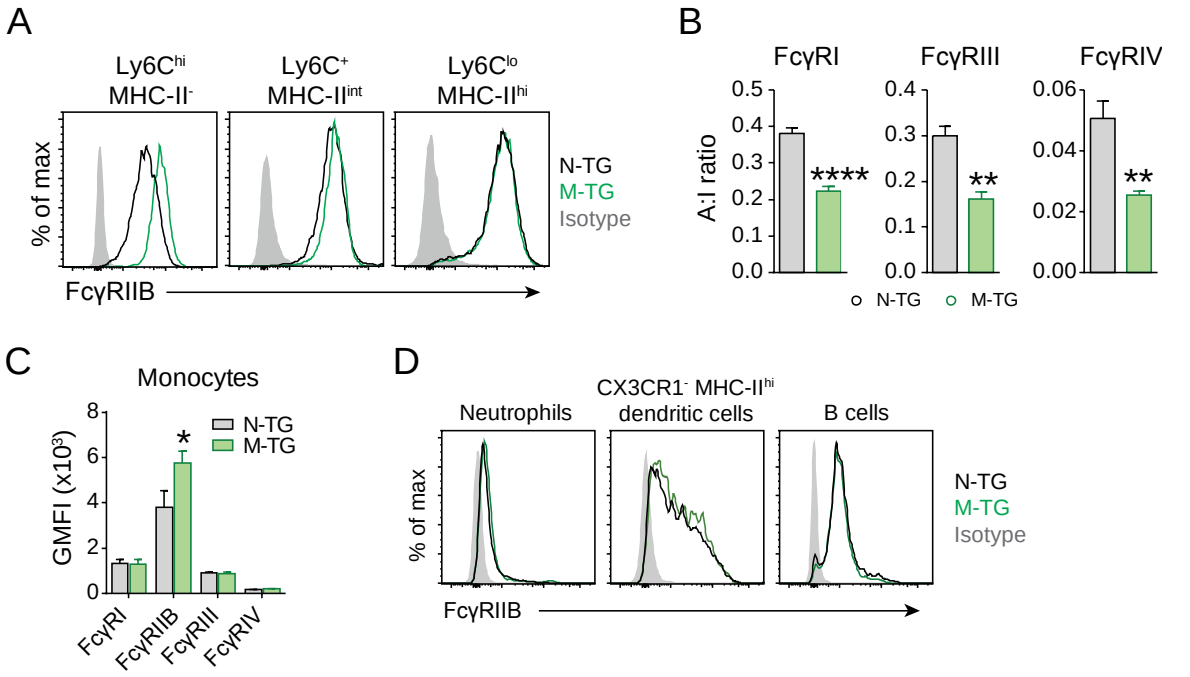

Figure 4.24. Reduced A/I ratios of intestinal CX3CR1^{+} MNPs in M-TG mice. (A) Flow cytometry staining of FcγRIIB in intestinal CX3CR1^{+} MNP subsets. (B) Quantification of monocyte A/I ratios from flow cytometry FcγR staining intensities. (C) Quantification of all FcγRs on intestinal monocytes in M-TG and N-TG mice. (D) FcγRIIB expression on other intestinal immune cell subsets known to express FcγRIIB. Data are representative of two independent experiments. *P* values were calculated using the parametric Students *t* test. * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$.

Opposite to the observed phenotype of *Fcgr2b*-deficient mice, M-TG animals lost less weight compared to littermate N-TG controls over the course of DSS-induced colitis, evident from as early as 7 days post-DSS administration (Fig. 4.25A). On day 21 of the chronic DSS protocol, M-TG animals demonstrated increased colon length and reduced colon weight, consistent with reduced severity of colitis (Fig. 4.25B). Furthermore, while splenic weight was relatively unchanged, MLN enlargement was reduced in M-TG animals, suggesting a localised reduction in inflammation in M-TG mice, unlike the more widespread immune dysregulation observed with *Fcgr2b*-deficient mice (Fig. 4.25C).

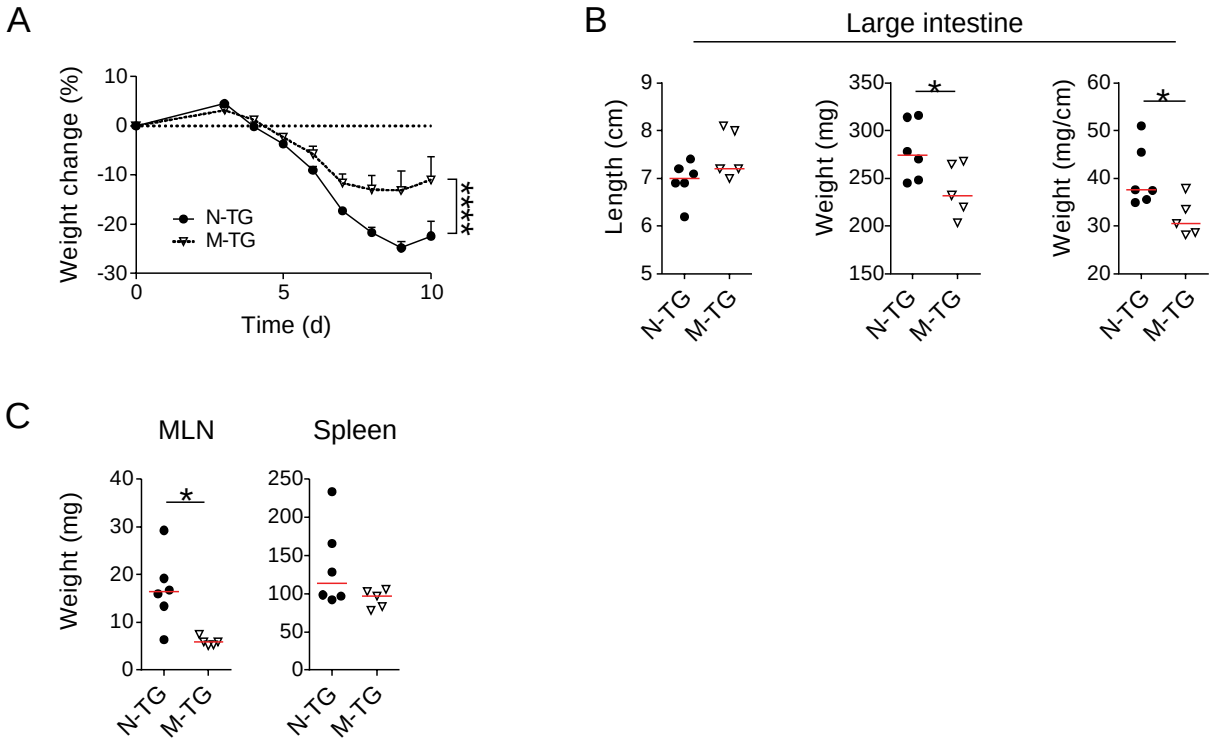

Figure 4.25. Reduced clinical severity of chronic intestinal inflammation in M-TG mice. (A) Weight loss in N-TG and M-TG mice following DSS administration. *n* = 6 per group. (B) Colonic clinical parameters in N-TG and M-TG mice following cDSS administration. (C) MLN and spleen size in N-TG and M-TG mice following cDSS. Data are representative of three independent experiments. *P* values were calculated using a two-way ANOVA (A) and the nonparametric Mann-Whitney test (B, C). * $P < 0.05$; **** $P < 0.0001$.

4.5.2. Inflammatory cytokine and chemokine analysis

qPCR analysis of global cytokine transcript levels demonstrated a specific reduction in *Il1b* levels (Fig. 4.26), with a smaller effect on *Tnf*, *Il23a*, *Il6*, and *Il10* (not shown), supporting the hypothesis that modulation of FcγRIIB expression can directly influence IL-1β-driven chronic colitis. While not significant, there was also trends towards the global reduction of *Cxcl1* in M-TG mice.

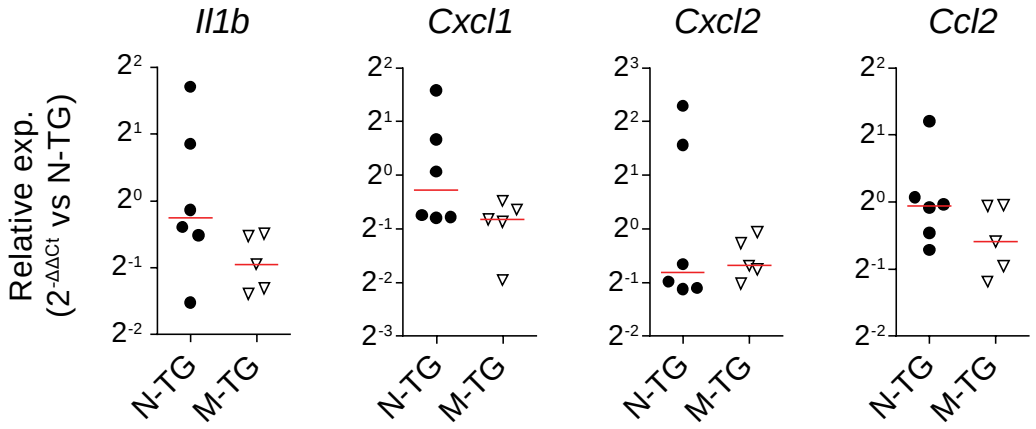

Figure 4.26. Reduced IL-1β production is a prominent feature of M-TG mice. qPCR of cytokines and chemokines in N-TG and M-TG whole colonic tissue following cDSS administration. Data are representative of two independent experiments.

Pro-IL-1β expression by CX3CR1^{+} MNP subsets was interrogated flow cytometry (Fig. 4.27). Strikingly, MNP-specific FcγRIIB overexpression significantly reduced the frequency (Fig. 4.27A) and absolute number (Fig. 4.27B) of pro-IL-1β^{+} colonic MNP subsets following chronic DSS-induced colitis, opposing the phenotype in *Fcgr2b*-deficient mice.

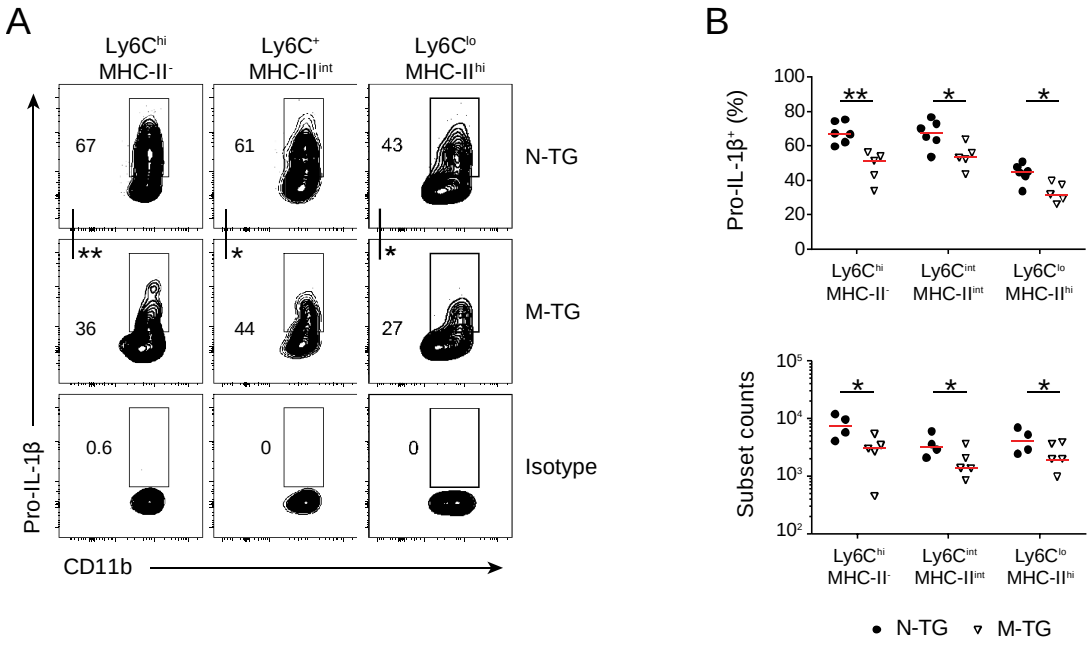

Figure 4.27. Reduced IL-1β production by intestinal CX3CR1^{+} MNPs in M-TG mice. (A) Flow cytometry of pro-IL-1β production by colonic CX3CR1^{+} MNPs in N-TG and M-TG mice following cDSS. (B) Quantification of frequency (top) and number (bottom) of pro-IL-1β^{+} CX3CR1^{+} MNP subsets. Data are representative of two independent experiments. *P* values were calculated using the nonparametric Mann-Whitney test. * $P < 0.05$; ** $P < 0.01$.

4.5.3. Leukocyte profiling in M-TG mice

Whereas *Fcgr2b*-deficient mice were characterised by enhanced colonic neutrophil and monocyte infiltration compared to WT mice, M-TG mice exhibited reduced neutrophil infiltration (Fig. 4.28A), a global reduction in total CX3CR1^{+} MNPs (Fig. 4.28B), and a reduction of all CX3CR1^{+} MNP subsets (Fig. 4.28C, D), dominated by a reduction in colonic monocyte infiltration compared to N-TG mice at day 21 post DSS administration. Analysis of splenic myeloid populations demonstrated a trend towards a reduction in monocytes in M-TG mice, but little change in systemic neutrophil levels compared to N-TG, suggesting that the genotype effects on neutrophil biology are localised to the colon and recruitment to the tissue out of the circulation (Fig. 4.28E). Alternatively, neutrophil retention in the colon may differ between M-TG and N-TG mice.

Unlike *Fcgr2b*-deficient mice, analysis of total colonic adaptive lymphocyte numbers by flow cytometry demonstrated no change in all CD3ε^{+} T cell subsets analysed or CD19^{+} B cells with genotype (Fig. 4.29A, B). IL-17A production was proportionally highest within γδ T cells, with approximately 10 % IL-17A^{+} (Fig. 4.29C). IL-17A production was also observed within CD4^{+} αβ T cells, and less so in CD4^{-} αβ T cells. Notably, in M-TG animals, IL-17A production was significantly reduced within both γδ T cells and CD4^{+} αβ T cells in the LP. Little change was see in CD4^{-} T cells. This resulted in a reduction in the absolute number of IL-17A^{+} T cells in the inflamed colonic LP.

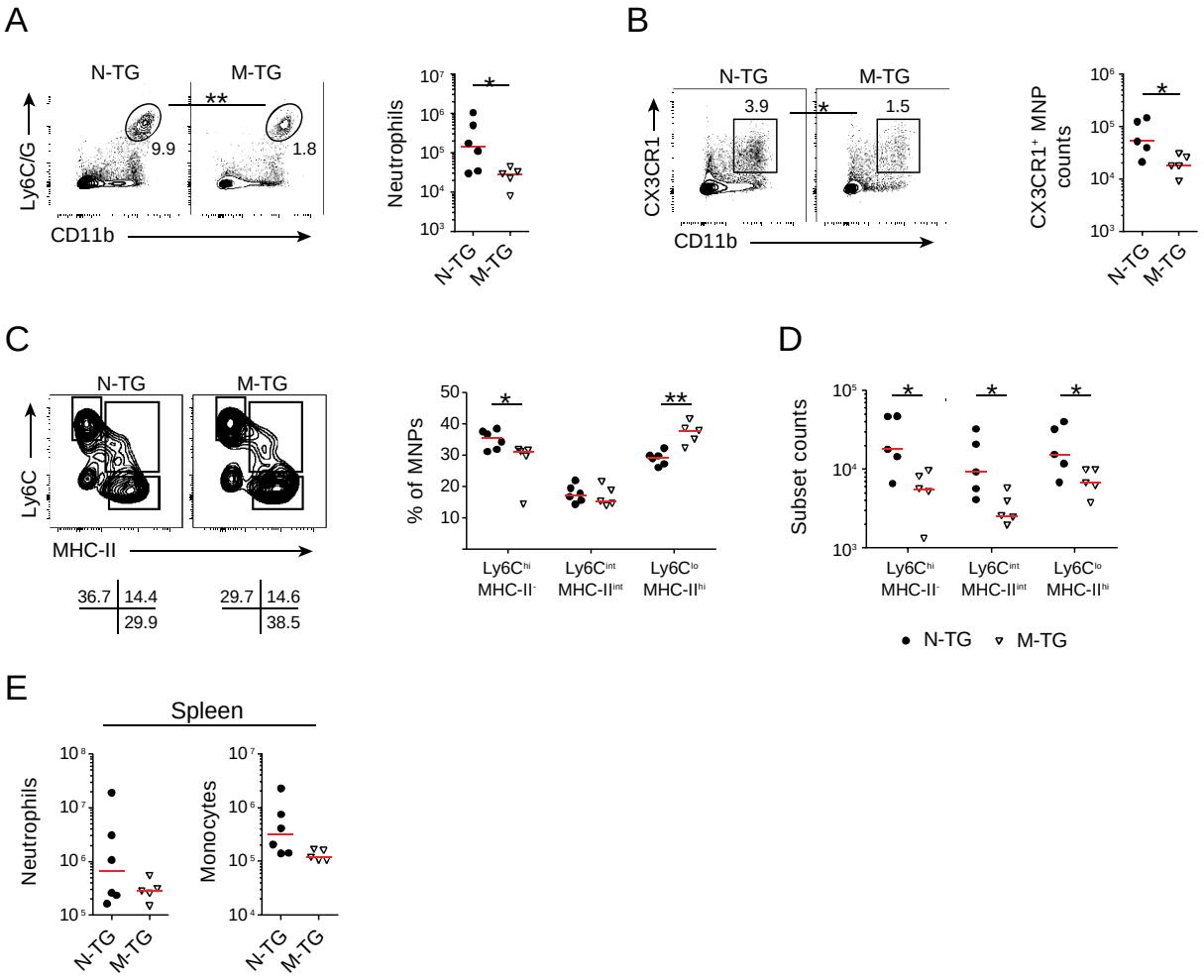

Figure 4.28. Reduced neutrophil and CX3CR1⁺ MNP numbers in M-TG mice. (A) Flow cytometry of colonic neutrophil infiltration in N-TG and M-TG mice following cDSS. (B) Flow cytometry of total colonic CX3CR1⁺ MNPs following cDSS. (C) Quantification of colonic monocyte waterfall frequency following cDSS. (D) Quantification of N-TG and M-TG colonic CX3CR1⁺ MNP subset numbers following cDSS. (E) Splenic neutrophil and monocyte quantification following cDSS. Data are representative of two independent experiments. *P* values were calculated using the nonparametric Mann-Whitney test. * $P < 0.05$; ** $P < 0.01$.

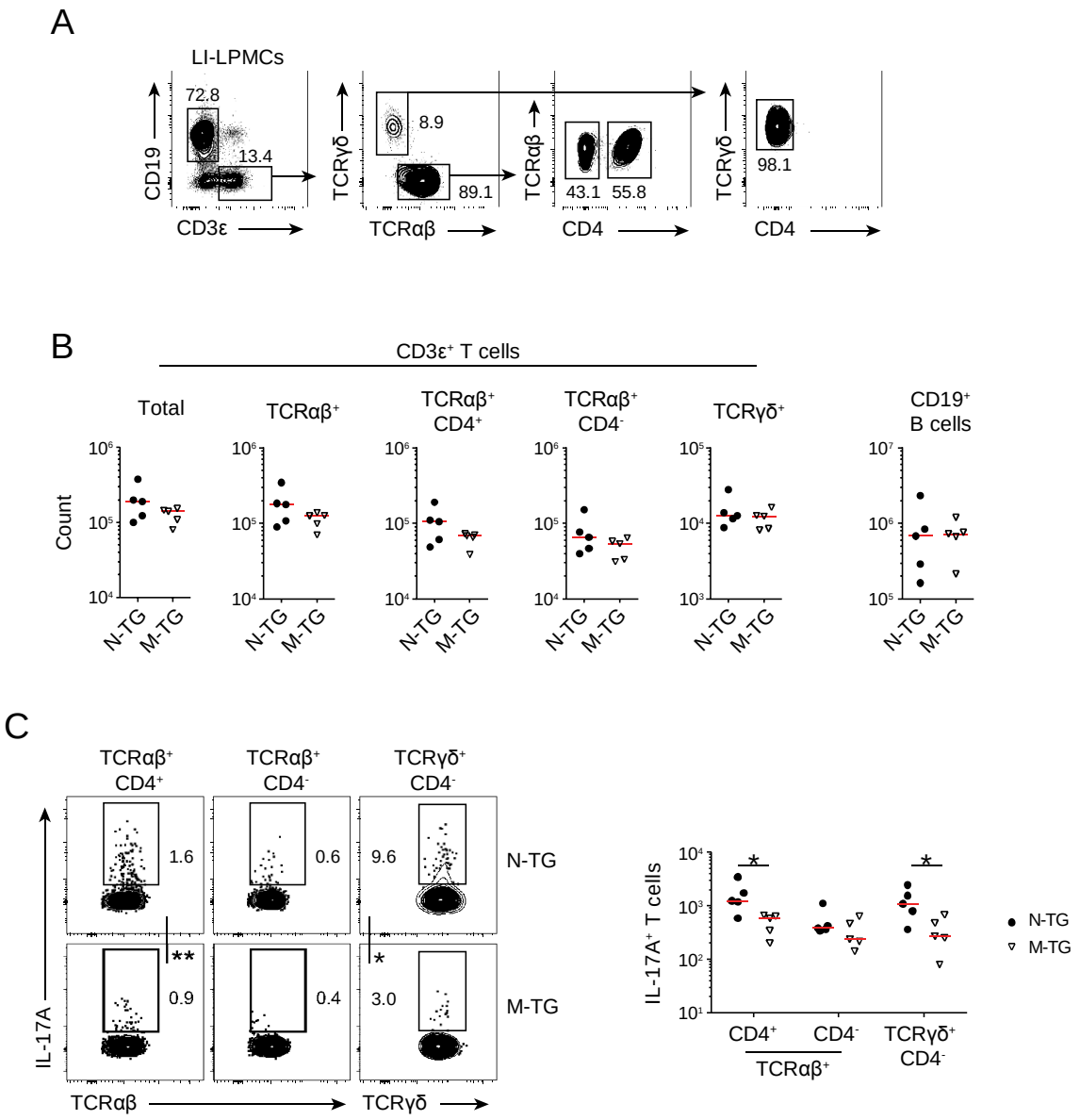

Figure 4.29. Impaired colonic Th17 immunity in M-TG mice. (A) Flow cytometry of colonic lymphocyte subsets following cDSS. (B) Quantification of lymphocyte subsets shown in A in N-TG and M-TG mice following cDSS. (C) Colonic Th17 T cell subsets in N-TG and M-TG mice. Data are representative of two independent experiments. *P* values were calculated using the nonparametric Mann-Whitney test. * $P < 0.05$; ** $P < 0.01$.

In summary, M-TG mice exhibit a reduction in chronic intestinal disease severity compared to N-TG littermate controls, opposing the phenotype see in *Fcgr2b*-deficient mice. Specifically, M-TG mice were characterised by reduced local MNP-derived IL-1β production, colonic monocyte and granulocyte infiltration, and a reduction in intestinal IL-17A-producing T cell subsets and demonstrate that modulation of the FcγR A/I ratio in intestinal macrophages is sufficient to alter disease progression via an IL-1β-dependent inflammatory network.

4.6. Discussion

4.6.1. Immune complexes promote IL-1β production by intestinal macrophages

Tissue-resident macrophages play essential roles in homeostasis, physiology, and inflammation, including within the GI tract [3]. Here, they are continuously derived from circulating monocytes [280], [487] and receive local cues from the environment and tissue-resident immune cells that direct their development to a predominantly scavenging, anti-inflammatory phenotype [425], [492], [497], [499], [500], [504]. During inflammation, however, monocytes are directed towards a pro-inflammatory phenotype, with elevated production of IL-1β, IL-23, and chemokines, all of which have been demonstrated to drive intestinal inflammation in numerous murine models [82], [279], [293], [295], [516], [620]. How production of these inflammatory mediators is regulated, however, is poorly understood.

We set out to investigate the effect of IC signalling on IL-1β production by intestinal macrophages. Here, we have shown that FcγR cross-linking has a profound transcriptional reprogramming of BMDM and flow-sorted intestinal macrophages, with several inflammatory pathways upregulated in the presence of IgG. Central to this was the induction of cytokines associated with UC pathology, including IL-1β, IL-23, and neutrophil-recruiting chemokines CXCL1 and CXCL2 (and CXCL8 in humans).

These results support observations in the previous chapter that IC could induce IL-1β and chemokine production by LPMCs at the RNA and protein levels. In addition to IL-1β and IL-23, we also observed an increase in *Spp1*, encoding osteopontin, *Il6*, and *Il12b*. Although not all of these genes are associated with UC pathogenesis, all are implicated in the activation of Th17 immunity, and demonstrate this as a predominant function of FcγR signalling in intestinal MNPs. Given their predominant location within the LP and non-migratory phenotype, FcγR-induced macrophage-derived cytokines may support the maintenance or enhanced pathogenicity of Th17 within the mucosa following generation in local GALTs. As macrophages and DCs exhibit similar cytokine profiles in the gut, as well as prominent FcγR expression, it is tempting to speculate that FcγR ligation on DCs may drive CCR7-dependent migration to and Th17 induction within local draining MLNs [88]. However, we have not investigated this here.

Other than Th17-inducing cytokines, we observed an increase in *Tnf* and *Osm* transcripts. Recently, Powrie and colleagues demonstrated a role for OSM in the activation of intestinal stromal cells in infliximab-refractory IBD, although the source of this cytokine was not investigated [334]. These results suggest that in the context of chronic inflammation, local IgG may promote a cooperative OSM/TNF-driven inflammatory response via their action on local non-haematopoietic cells. IL-10 expression was also significantly induced by IC. This may represent a negative feedback loop to prevent excessive inflammation, consistent with the role of this cytokine in preventing IBD-associated pathology. Preferential engagement of

inflammatory FcγR-induced cytokines in the *H. hepaticus* + anti-IL-10R IgG model of colitis may contribute, therefore, to some of the detrimental inflammatory mechanisms in this model, although this has yet to be investigated.

Transcriptomics analyses has also yielded insights into the potential mechanisms that regulate IL-1β secretion by intestinal macrophages in response to FcγR cross-linking. IC induced expression of NFκB components and *Hif1a*, two TFs known to drive expression of IL-1β and NLRP3 genes, the latter a component of the canonical inflammasome. HIF-1α has been shown to directly drive IL-1β expression in macrophages during cellular glycolytic reprogramming and the accumulation of intracellular succinate [343]. Whether or not FcγR signalling may similarly regulate macrophage metabolism has not been investigated here, but is currently the subject of research within the lab. Therefore, FcγR signalling could prime macrophages for assembly of the inflammasome, drive a switch to glycolysis, and directly induce expression of pro-IL-1β. How FcγR may cooperate with secondary signals for IL-1β secretion is not clear. IC stimulation of BMDMs only induces minor mature IL-1β secretion, but this signal is much greater in LPMCs stimulated with IC. It is possible that the nature of intestinal macrophages, compared to BMDMs, promotes significant IL-1β secretion in the absence of secondary signals. Alternatively, secondary stimuli may be present in LPMC cultures, as yet to be identified, that promote pro-IL-1β cleavage. For example, GM-CSF can induce IL-1β secretion in response to LPS via induction of glycolysis and GTPases required for processing. What appears to be clear from these analyses, however, is that FcγR signalling has a direct effect on the induction of IL-1β production, rather than inducing secondary signals from macrophages that promote basal pro-IL-1β cleavage, although we cannot rule out that this may have an effect. Regardless, these results are contrary to reports that FcγR signalling acts to antagonise assembly of the inflammasome [79], and illustrate the requirement to analyse macrophages *ex vivo*.

The characteristic milieu of the intestine may induce a macrophage phenotype that resembles more closely M2 macrophages. In humans, M2 macrophage FcγR stimulation drives Th17 induction, but not in M1 macrophages [74]. Therefore, FcγRs could be particularly important in the reprogramming of “anti-inflammatory” tissue-resident macrophages to a more pro-inflammatory state in the context of a local IgG response. Indeed, as IgG is very low at mucosal sites during homeostatic conditions, this could potently act as an alarmin to skew intestinal immune responses. This has potential implications for UC and CD, with whom the FcγRIIA-H/R131 variant exhibits differential association. CD is associated with elevated IFNγ production and a M1-like macrophage phenotype. In contrast, UC is associated with elevated IL-4 production, which may support an M2-like macrophage phenotype. In the latter, local FcγR ligation on tissue-resident macrophages may have a more profound effect, driving macrophage polarisation towards IL-1β and IL-23 production, the induction of a Th17

response, and a chronic inflammatory state. Whether this is the case is unknown, but may be addressed by the addition of additional secondary signals to inflamed LPMCs, including IFNγ. It is noteworthy that DSS-induced colitis is thought to more closely resemble UC-associated pathology, making a useful model to study FcγR-associated phenomena. Throughout this study, we have primarily used O-IC as a model of FcγR stimulation due to its inability to ligate TLRs that may confound results. However, it would be of interest to determine whether inflammatory responses following TLR-FcγR co-stimulation on intestinal macrophages exhibit a different profile to FcγR engagement alone.

4.6.2. Enhanced DSS-induced colitis severity in the absence of FcγRIIB

We have shown that macrophage-intrinsic FcγR signalling has the potential to drive intestinal inflammation through the induction of Th17-associated immune networks. Currently, we are in the process of obtaining PBMC samples from individuals with different FcγRIIA variants in order to grow MDMs and address whether the magnitude of IL-1β responses *in vitro* is dependent on genotype. This would help to clarify whether this may represent a relevant network contributing to UC susceptibility in these individuals: individuals with the UC-associated FcγRIIA-H131 high-affinity variant may be at increased risk of exacerbated IL-1β-driven pathology as a result of increased macrophage FcγR A/I ratios.

To test this hypothesis, we first sought to investigate the DSS-induced disease course in *Fcgr2b*-deficient mice. These mice lack the inhibitory FcγR and are susceptible to autoimmune and inflammatory disorders, but demonstrate increased resistance to infectious agents. We demonstrated enhanced clinical features of inflammation in *Fcgr2b*-deficient mice following chronic DSS administration, including weight loss, SLO enlargement, and leukocytic infiltration. These mice exhibited an inflammatory phenotype consistent with IC-inducible cytokines in macrophages, with elevated global and MNP-intrinsic IL-1β and chemokine levels compared to WT mice, resulting in increased neutrophil and monocyte infiltration into the colon. Strikingly, blockade of IL-1β signalling significantly improved several features of inflammation, including weight loss and colonic neutrophil infiltration. Furthermore, *Fcgr2b*-deficient mice exhibited elevated T cell-derived IL-17A and IL-22 production that was dependent on IL-1β. In particular, FcγR signalling appears to have profound effects on cytokine production by γδ T cells, a population of T cells known for their innate production of IL-17A in response to IL-1β stimulation [354], [355].

As previously noted, IL-17A neutralisation itself is detrimental in DSS-induced colitis and human IBD, thought to be driven by effects on the epithelial barrier [385]–[389]. IL-22 has also been shown to contribute to barrier homeostasis [361], [399]–[402]. Therefore, readouts of Th17 immunity in this case are likely a surrogate marker for the function of IL-1β within the GI tract, rather than being implicated in driving IL-1β-dependent inflammation. This is not to say

that these cytokines may not be involved. IL-17A is known to promote neutrophil recruitment and monocyte activation within inflamed tissues, and its blockade is beneficial in numerous circumstances [366], [367], [378], [381], [383]. It is possible that, in *Fcgr2b*-deficient mice, exacerbated IL-17A is pathogenic via similar mechanisms. However, its complete neutralisation may also be detrimental. Rather, therapeutic strategies may be better placed by modulating IL-17A expression so as to return it to within normal ranges. Future approaches may involve analysing IFNγ and GM-CSF production by Th17, γδ T cells, and ILC3s. Certainly, IFNγ is implicated in driving intestinal inflammation: its expression is significantly increased in CD and its blockade is beneficial in several murine models. However, this has not been investigated here. It is possible that FcγR-driven IL-1β-dominated milieu may drive the emergence of pathogenic dual-IFNγ/IL-17A-expressing Th17 cells that contribute to intestinal pathology, and may suggest why IL-1β blockade is particularly effective. The role of GM-CSF is less clear. While its expression is increased in colitis and may contribute to granulocyte recruitment and activation, its effects on regulating tolerogenic DCs, coupled with observations of anti-GM-CSF antibodies in IBD patients, demonstrate significant homeostatic functions for this cytokine within the GI tract. It is possible that IL-1β and IL-23 blockade are more effective in colitis due to their action on several Th1 and Th17 cytokines. Alternatively, non-T cell-mediated effects, such as the pyrogenic and neutrophil-activating properties of IL-1β, may be involved in driving inflammation. Alternatively, disease may reflect a combination of all of these factors.

These results raise the question once more as to how FcγR-induced IL-1β and type 17 immunity may feed backwards to regulate the IgG response? Could ongoing detrimental IL-1β-driven inflammation promote the emergence of pathogenic IgG subclasses and altered glycosylation profiles, for example?

Clearly, however, these results demonstrate, for the first time, a link between FcγR signalling, IL-1β, and mucosal Th17 immunity, and resemble immunological features of *Fcgr2b*-deficient mice in other organ systems, such as the kidney [183]. *C. rodentium* infection is often used in the study of intestinal Th17 responses and requires IgG and FcγR signalling for sterilising immunity [283], [473], [523]. Therefore, it would be of interest to investigate the infection resistance and immune pathology of *Fcgr2b*-deficient mice following *C. rodentium* challenge, as one might expect a similar FcγR-inducible IL-1β-dependent IL-17A network.

Despite these results, certain caveats exist in this study, both from the use of *Fcgr2b*-deficient mice and blockade strategies. For IL-1β blockade *in vivo*, a blocking anti-IL-1R1 IgG monoclonal antibody was used. This receptor is shared by IL-1β and IL-1α, the latter also being induced by IC signalling on macrophages. As such, we cannot rule out that IL-1R1 blockade has IL-1β-independent effects in the amelioration of disease. Furthermore, given the widespread expression of this receptor, it is unclear whether the dosing used here was

sufficient to completely block IL-1-mediated signalling. To resolve this question, *Fcgr2b*-deficient mice could be crossed to *Il1b*-, *Nlrp3*-, or *Casp1/4*-deficient mice to abrogate this pathway.

It is of note that certain clinical features were not significantly different in *Fcgr2b*-deficient mice. For example, colon length is widely used as a measure of inflammation, but was relative unchanged here [264], [621]. Given the effect of IC on the induction of anti-inflammatory genes, such as IL-10, it seems likely that several pro- and anti-inflammatory mechanisms contribute to the dysregulated immune response in these mice. The balance of these mechanisms may contribute to the worsening of certain clinical features but not others. Histological analyses have not been carried out here, but remain a priority for future experiments to determine the extent to which these mouse models affect tissue destruction.

As FcγRIIB is most highly expressed on gut macrophages, this represents a reasonable model to study FcγR signalling on macrophages, with immunopathology mirroring cytokine profiles identified *in vitro*. However, FcγRIIB is widely expressed on both haematopoietic and non-haematopoietic cells. Therefore, receptor deficiency is likely to affect numerous immunological mechanisms. Indeed, analysis of the B cell phenotype in these mice demonstrates an overactive B cell compartment within SLOs after DSS administration, and may support the enlargement of these organs. While these observations may be relevant to individuals with polymorphisms causing FcγRIIB loss-of-function, they are unlikely to arise solely from macrophage-intrinsic effects. As *Fcgr2b*-deficient mice age, they develop autoimmune IgG antibodies and autoimmunity, consistent with roles in B cell tolerance [62]. It is likely that *Fcgr2b*-deficient mice also generate higher titres of anti-commensal IgG antibodies that may contribute to intestinal pathology. For example, increased bacterial opsonisation in *Fcgr2b*-deficient mice could drive elevated IL-1β expression in the absence of differences in macrophage A/I ratios, confounding observations.

Furthermore, *Fcgr2b*-deficient strains used here are derived from backcrossing *Fcgr2b*-deficient mice on the 129-background, and as such contain additional susceptibility loci. While these loci augment disease severity, the immune mechanisms contributing to inflammation are largely similar to B6-generated *Fcgr2b*-deficient mice [194]. Therefore, while useful in illustrating the relevance FcγR mechanisms to disease *in vivo*, confounding features in these mice do not allow for dissection of the contribution of macrophage-intrinsic FcγR signalling to intestinal inflammation. Furthermore, as these mice are prone to hyperactive immune responses, profiling of all mouse strains under uninflamed conditions (i.e. non-DSS) is essential to determine the extent to which these phenomena are inflammation-dependent or arise naturally as a consequence of the strain. This has not been done here. A clearer model may involve the generation of macrophage-targeted FcγRIIB depletion, such as a LysM-Cre or CD11c-Cre mechanism, although these are not without off-target effects. Alternatively, the

use of activating FcγR KO mice could help to support our observations, given their opposing phenotype to *Fcgr2b*-deficient mice in numerous models of IgG-driven inflammation [197], [199]–[201], [207], [208]. For example, FcγRIII or FcRγ-deficient strains may exhibit reduced IL-1β production within the chronically inflamed colon. However, use of these mice would have similar constraints to pan-FcγRIIB KO mice, and one might argue that ablation of activating FcγR signalling is not representative of the FcγR A/I ratio modulation observed in patients with IBD. Specifically, manipulation of the inhibitory receptor is more likely to mirror different strengths of activating signalling observed in individuals with the FcγRIIA-R/H131 variant, which bind IgG with different affinity. Some of these issues have been addressed through the use of M-TG mice, as will be discussed below.

It would also be of interest to develop a robust anti-commensal IgG transfer model analogous to that used by Blumberg and colleagues [482]. Are the IL-1β-dependent inflammatory mechanisms similarly induced by transfer of IgG? We have addressed this to a small extent using the *E. coli* IgG transfer model, but cellular characterisation and clinical features of inflammation are currently lacking.

4.6.3. M-TG mice are protected from DSS-induced colitis

Because of the limitations of *Fcgr2b*-deficient mice, we utilised M-TG mice as a means to specifically manipulate the macrophage A/I ratio, as detailed above. M-TG mice drive transgenic FcγRIIB expression via the CD68 promoter and have furthered our understanding of the role played by macrophages in IgG-driven disorders [205].

The results presented here demonstrate that M-TG mice are protected from overt inflammation in DSS-induced colitis. Compared to littermate N-TG controls, M-TG mice lost less weight, had improved clinical features of inflammation, and reduced neutrophil and monocyte infiltration into the colon. Furthermore, the immune phenotype in these mice was dominated by a reduction in IL-1β expression by intestinal MNPs and a corresponding decrease in IL-17A production by T cell subsets within the LP. Therefore, these results mirror those observed in *Fcgr2b*-deficient mice, and demonstrate the critical importance of macrophage-intrinsic FcγR signalling in mediating intestinal inflammation.

Unlike *Fcgr2b*-deficient mice, M-TG mice did not exhibit profound differences in splenic enlargement or neutrophil counts within this organ. This is consistent with the more generalised inflammatory defect observed in *Fcgr2b*-deficient mice, given its expression across a wide range of cells. Interestingly, macrophage-specific overexpression of FcγRIIB did affect MLN size. Although the reason for this is unclear, it possible that the reduced inflammatory milieu in these mice suppresses DC migration, lymphocyte activation and GC formation. However, as total T and B cell numbers within the LP are unaffected in M-TG mice, reduced IL-17A production may arise specifically from the reduction in mucosal Th17-

promoting cytokines rather than Th induction in local lymph nodes. Alternatively, FcγRIIB overexpression in M-TG mice has been shown to suppress VEGF-A-mediated enlargement of SLOs in response to IC [622]. A similar effect may be occurring here.

In future, more targeted attempts to model the exact contribution of the FcγRIIA-H/R131 variant on susceptibility to IgG-mediated intestinal pathology are required, but were beyond the scope of this PhD. For example, the use of humanised mouse models expressing FcγRIIA in combination with other receptors or alone may help to determine the magnitude of the contribution of this receptor to disease. Furthermore, the generation of transgenic mice expressing the exact variants of FcγRIIA would help to take these observations one step further. Both approaches would go a considerable way to overcoming the shortfalls of using mutant FcγRIIB mice to address signalling from an activating receptor, as this will likely have off-target effects on other FcγRs other than FcγRIII (mouse homologue of FcγRIIA), as well as potentially other signalling pathways, as discussed in the Introduction chapter.

4.6.4. Summary

An outstanding question in these studies is why does FcγR signalling affect IL-1β signalling so dramatically, as opposed to other inflammatory cytokines induced by FcγR cross-linking *in vitro*? For example, exogenous stimulation of macrophages induces IL-23 in addition to IL-1β. However, LPMCs do not induce IL-23 in response to IgG and little change is observed between *Fcgr2b*-deficient, WT or M-TG in their expression of IL-23. Furthermore, effects on TNF are relatively smaller compared to flow-sorted macrophages. There may be several explanations for this. IL-1β expression is highest within the inflamed intestine and in flow-sorted macrophages, compared to other cytokines. Therefore, modulation of this cytokine is likely to have a dominant effect on inflammation. Alternatively, IL-1β is known to be directly induced by commensal microbes and further augmented by opsonisation, at least in human macrophages [74], [279]. The unique integration of FcγR and TLR signals may preferentially boost certain pathways over others, such as IL-23. This is consistent with a study by Uo et al. in which only TLR stimulation had a more pronounced effect on IL-23 expression [430]. In our studies, most FcγR cross-linking studies were carried out with model ICs made of OVA, which do not engage other PRRs. Therefore, it is possible that opsonised bacteria elicit a different magnitude or response profile, compared to FcγR cross-linking alone, which is more representative of inflammation *in vivo*. Indeed, classical mature IL-1β production requires two steps. However, Seo et al. demonstrated that certain commensal species contain sufficient signals to induce IL-1β secretion directly, unlike LPS alone. Could opsonisation of commensal microbes, therefore, potentiate this mature IL-1β secretion in a manner difficult to model *in vitro*? It seems clear that, despite our understanding of the major players involved in inflammasome assembly and activation, how these processes are regulated is not well known. Alternatively, as previously alluded to, the inflammatory mechanisms that underlie a particular

IBD model also influence the potential effect of FcγR signalling pathways. For example, CD models, in which IFNγ plays a more major role, may drive alternative mechanisms of inflammation that revolve around Th1 activation.

FcγRs have been shown to bind components of innate humoral immunity, such as pentraxins, as well as cross-link to other non-Fcγ receptors on the cell surface. While anti-flagellin IgG transfer has been demonstrated to be pathogenic in DSS-induced colitis, our results do not rule out the contribution of non-IgG-mediated effector functions of FcγRs in colitis. For example, FcγRIIB may cross-link to TLRs. Therefore, FcγRIIB deficiency may result in elevated TLR-driven IL-1β production. These effects would be unrelated to those linked to the UC-associated *FCGR2A* polymorphism. Additionally, the expression of FcγRIIB on myeloid progenitor cells in the bone marrow and the role of this receptor in the apoptosis of bone marrow-resident plasma cells may contribute to systemic extra-intestinal effects in these mice, such as monocytosis [106], [194]. Furthermore, certain pathological features *in vivo* could be driven by FcγRs in the absence of IgG: binding to CRP or SAP could stimulate inflammatory cytokine production within the LP [63], [117]–[120]. Certain studies may help to resolve these issues. Direct stimulation of intestinal or *in vitro*-derived macrophages with IgG opsonised commensal bacteria in the presence or absence of FcγR-blocking antibodies may help to ascribe functionality to each component. For example, FcγRIIB blockade in the presence of unopsonised bacteria may result in enhanced inflammatory cytokine production if FcγRIIB regulates signalling via IgG-independent pathways.

In conclusion, the results presented in this chapter demonstrate that FcγR signalling can control inflammatory cytokine production by intestinal macrophages and monocytes both *in vitro* and *in vivo*. This effect is dominated by IL-1β production, a prototypical inflammatory cytokine associated with chronic inflammation in human IBD and murine models of colitis. A/I ratio manipulation on macrophages is sufficient to dictate IL-1β-dependent inflammation, as determined by classical clinical features of inflammation and mucosal Th17 activation, and targeting this pathway is beneficial in DSS-induced colitis. These results, therefore, identify FcγR signalling as a major determinant of colitis severity, which may be targeted to improve disease progression (Fig. 4.30).

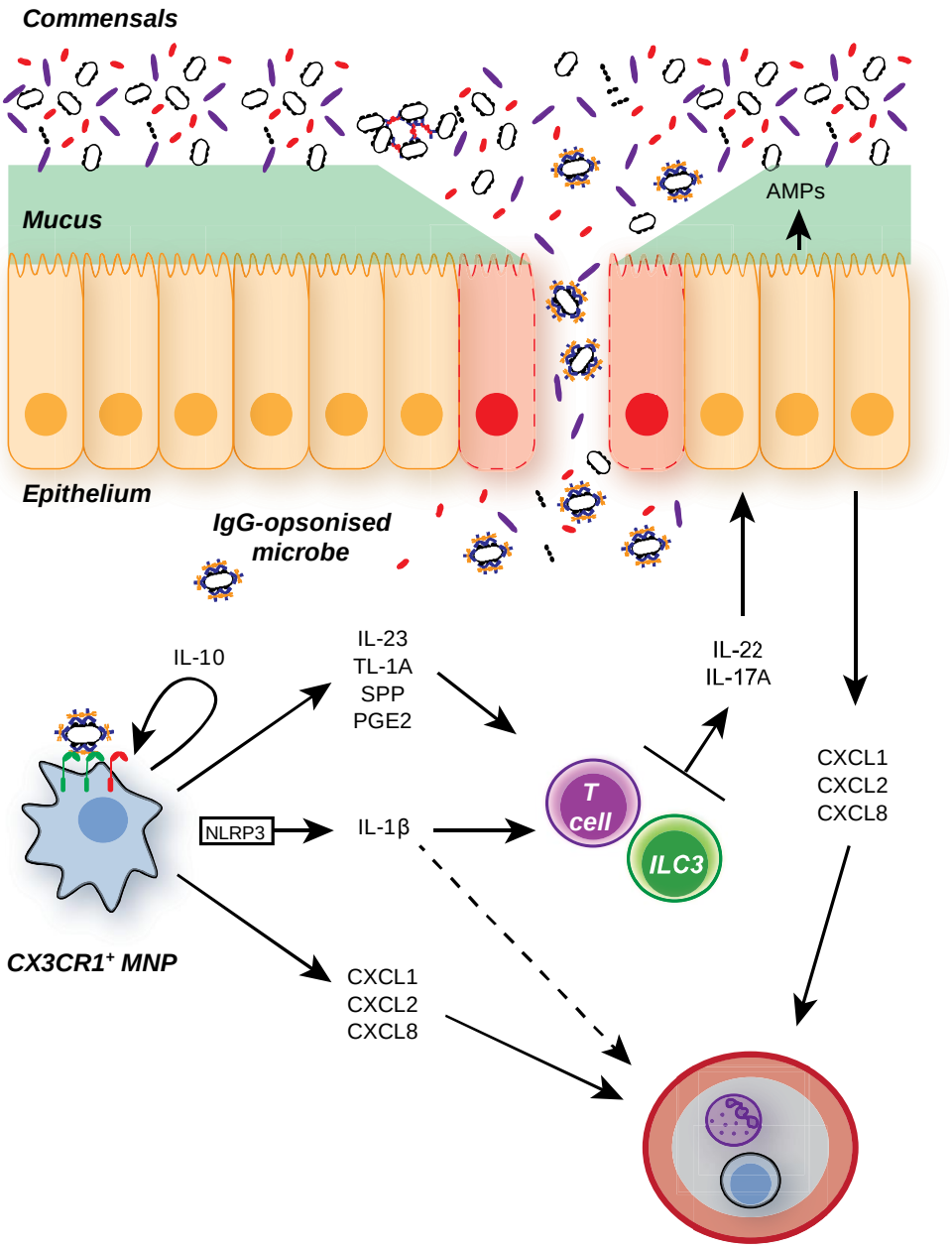

Figure 4.30. Chapter 4 graphical summary. FcγR signalling drives the inflammatory activation of intestinal macrophages. IC induces a transcriptional programme dominated by UC-associated cytokines, including IL-1β, IL-23, and TNFα. IC stimulation also results in the production of neutrophil recruiting chemokines. In vivo, manipulation of this inflammatory network by transgenic alteration of the macrophage A/I ratio is sufficient to alter the progression of intestinal inflammation, the induction of mucosal Th17 cells, and neutrophil recruitment. Green receptor = activating FcγR; red receptor = inhibitory FcγRIIB. AMP = anti-microbial peptides.

5. Expression and function of Fcγ receptors on group 3 innate lymphoid cells

5.1. Introduction and hypotheses

We have previously shown that *Fcgr2b*-deficient mice exhibit enhanced mucosal IL-1β-dependent Th17 immune responses, with an increase in IL-17A and IL-22-producing T cell subsets within the colonic mucosa. We also observed global increases in *Il17a*, *Il22*, and *Csf2* expression levels within the inflamed colon by RNA analysis. Other than T cells, ILC3s are a major source of these cytokines within the GI tract. ILC3s are innate Th17-like cells enriched at mucosal sites, particularly the GI tract, that produce type 17-associated cytokines in response to cytokine stimulation [12], [525]. In particular, MNP-derived cytokines, including IL-1β, IL-23, and TL1A, are critically implicated in regulating ILC3 biology *in vivo* [290]–[292], [359].

Little is known about how FcγR signalling may regulate the functional phenotype of these cells, either *in vitro* or *in vivo*. Firstly, FcγR-inducible cytokines produced by macrophages and DCs, as highlighted in the previous chapters, may regulate ILC3 activity indirectly. Secondly, given their close developmental relationship with NK cells, it remains unexplored whether helper ILC subsets similarly express FcγRs. Murine NK cells express FcγRIII, the canonical low-affinity activating FcγR, while in humans, NK cells express FcγRIIIA (and FcγRIIC in certain circumstances) [102], [103]. In these cells, FcγR signalling mediates IFNγ production and ADCC, the directed release of cytotoxic molecules and perforin at IgG-opsonised cells for target cell killing [104], [105]. Therefore, FcγR signalling drives canonical functions in these cells. This raises the question whether FcγR signalling may control cytokine production or antigen presentation by ILCs. Murine ILCs in particular are well known for their absence of PRRs and demonstrates a key feature of ILC biology: the inability to directly sense danger-associated molecules. However, if ILCs were to express FcγRs, this would represent a shift in our understanding of these cells. In circumstances of IgG opsonised antigens, such as in chronic inflammation or a memory B cell response, ILCs would be able to react directly, without the requirement for secondary microbe-sensing cells, and highlighting a novel mechanism of innate-adaptive communication.

Here, we sought to investigate the FcγR expression profile of helper ILC subsets within the GI tract and extra-intestinal tissues. We hypothesised that ILCs would express FcγRIII, similar to NK cells, with FcγR cross-linking potentially driving enhanced cytokine production, antigen presentation, and migration.

5.2. Profiling FcγR expression on innate lymphoid cells

To first address this question, we analysed small intestinal ILC transcriptomic datasets from the Immgen consortium (Fig. 5.1). ILC1s and all subsets of ILC3s expressed *Fcgr3*, similar to NK cells (Fig. 5.1A). Furthermore, NKp46$^+$ ILC3s expressed highest levels of FcγRIII. Minimal FcγRIII expression was detected on ILC2s. A similar expression pattern was seen for *Fcer1g*, the common γ-chain required for signalling from FcγRIII, and *Fgr2b*. FcγR expression was also analysed on flow-sorted intestinal and splenic RORγt$^+$ ILC3s by qPCR, and demonstrated a similar expression pattern, with highest levels of FcγRIII and the common γ-chain, and lower levels of FcγRIIB (Fig. 5.1B). Therefore, ILC3s and ILC1s express a similar FcγR expression pattern to NK cells in mice, both at mucosal and non-mucosal sites, with highest levels of the prototypical activating FcγRIII and associated adaptor signalling molecule, and lower levels of the inhibitory receptor. As such, they could potentially respond directly to local IgG ICs.

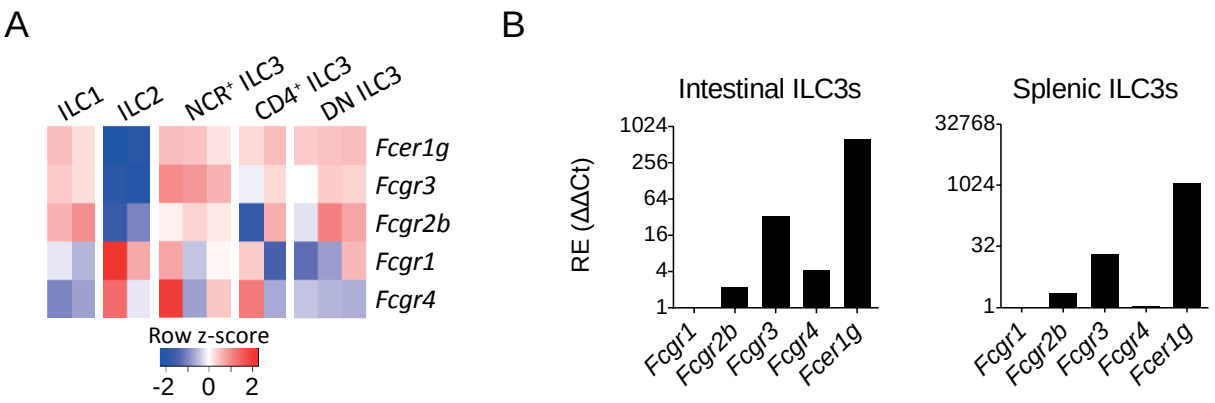

Figure 5.1. ILC3s and ILC1s dominantly express the prototypical activating FcγRIII. (A) Heatmap showing FcγR gene expression across different SI-resident ILC subsets in a transcriptomics dataset (GSE37448). (B) qPCR of FcγR expression in flow-sorted intestinal and splenic RORγt$^+$ ILC3s. Data are representative of two independent experiments.

Flow cytometry was used to profile FcγR expression on all helper ILC subsets in the SI. ILCs were identified as Lin$^-$ CD45$^+$ IL-7Rα$^+$. ILC3s were firstly identified as Lin$^-$ CD45^{int} IL-7Rα$^+$ RORγt$^+$ cells (Fig. 5.2A). Three ILC3 subsets were then identified based on expression of NKp46 and T-bet:

1. T-bet$^+$ NKp46$^+$
2. T-bet$^+$ NKp46$^-$
3. T-bet$^-$ NKp46$^-$

All three subsets expressed FcγRs, as determined by FcγRII/III staining using the 2.4G2 antibody clone (Fig. 5.2B), with T-bet$^+$ NKp46$^+$ ILC3s expressing the highest levels of FcγRs.

ILC2s were identified as RORγt$^-$ KLRG1$^+$ cells, while ILC1s could be identified as RORγt$^-$ KLRG1$^-$ T-bet$^+$, and further subdivided into NKp46$^+$ and NKp46$^-$ subsets (Fig. 5.2C). Interestingly, of the remaining ILC subsets, only the T-bet$^+$ NKp46$^+$ ILC1s expressed FcγRs, mirroring the expression pattern in ILC3 subsets and the known expression of FcγRIII by murine NK cells.

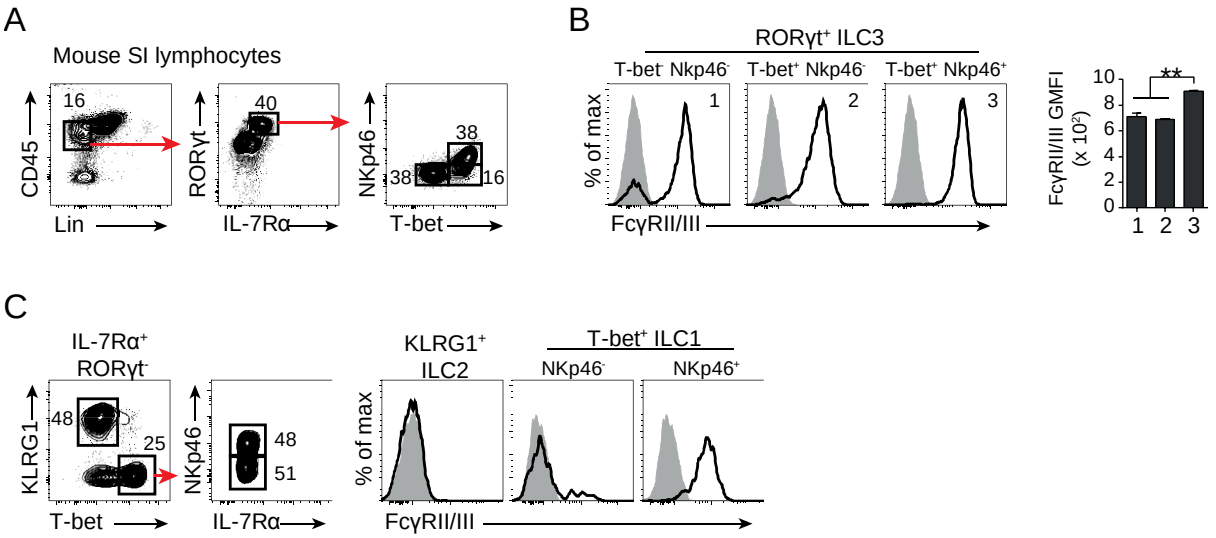

Figure 5.2. Intestinal ILC3s and ILC1s express Fcγ receptors. (A) Flow cytometric gating strategy for the identification of SI-resident ILC3 subsets based on NKp46 and T-bet expression. (B) ILC3 subset FcγR staining and quantification using the anti-FcγRII/III antibody clone 2.4G2. (C) Identification and FcγR expression by SI-resident KLRG1$^+$ ILC2s and T-bet$^+$ ILC1s. $n = 3$ per group. Data are representative of three independent experiments. P values were calculated using the parametric Student's t test. ** $P < 0.01$.

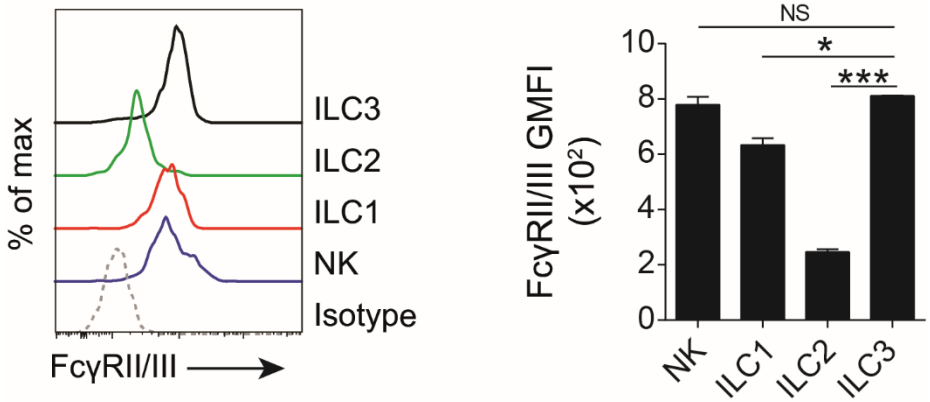

Figure 5.3. ILC3s express similar levels of FcγRs to NK cells. Flow cytometric quantification of FcγRII/III expression across small intestinal ILC subsets compared to NK cells. $n = 3$ per group. Data are representative of two independent experiments. P values were calculated using a one-way ANOVA. * $P < 0.05$; *** $P < 0.001$.

Comparison with NK cells demonstrated that SI-resident ILC3s had a very similar level of FcγR expression (Fig. 5.3), demonstrating an equal ability to respond to local IgG.

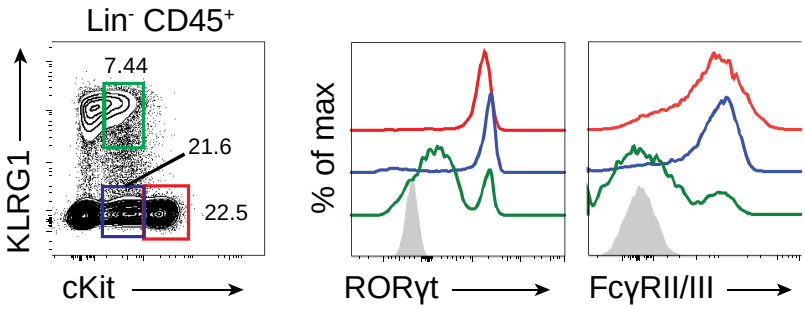

Figure 5.4. cKit-mediated ILC identification and FcγR expression. Flow cytometry showing ILC3 identification based on cKit expression for flow-sorting ILC3s from non-RORγt-GFP reporter mice. Data are representative of two independent experiments.

We confirmed by flow cytometry that cKit could be used to identify ILC3s, a strategy that would be essential for flow-sorting live cells from non-reporter mouse strains (Fig. 5.4). cKit^{int} KLRG1^{-} (blue) and cKit^{hi} KLRG1^{-} (red) subsets dually expressed RORγt and FcγRs, as determined by 2.4G2. A minority of cKit^{int} cells expressing KLRG1 also expressed RORγt, but otherwise was comprised predominantly of RORγt^{-} cells, consistent with ILC2s. This was confirmed by the absence of FcγR staining on these cells.

In addition to SI-resident cells, ILC3s and ILC1s expressed FcγRs across all murine tissues examined, including the colon, MLNs, and spleen (Fig. 5.5A, B). Therefore, IgG can potentially activate ILCs via FcγR ligation throughout the body, not just at mucosal surfaces.

In summary, we have demonstrated that T-bet^{+} NKp46^{+} ILC1s and all subsets of ILC3s express FcγRs, while ILC2s were devoid of FcγRs, as determined by flow cytometry. Furthermore, FcγR characterisation at the RNA level in mice demonstrated significant expression of FcγRIII and minimal expression of the inhibitory receptor, FcγRIIB, consistent with the expression pattern of NK cells. Therefore, ILC1s and ILC3s may potentially be activated by cross-linking cell surface FcγRs.

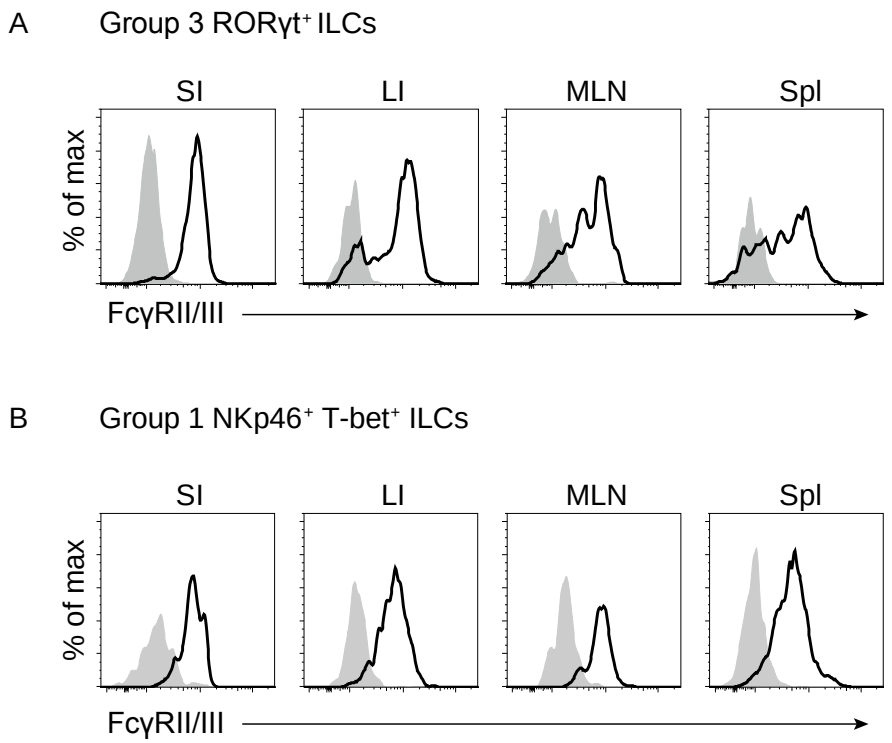

Figure 5.5. ILCs express FcγRs throughout the body. Flow cytometry showing FcγRII/III staining on Lin$^-$ IL-7Rα$^+$ RORγt$^+$ ILC3s (A) and NKp46$^+$ T-bet$^+$ ILC1s (B) in different organs. Data are representative of two independent experiments.

5.3. Immune complex stimulation of ILC3s *in vitro*

Given the enrichment within the GI tract, ease of identification by several strategies, and key roles in regulating immune responses within the colonic mucosa, we focused predominantly on the effect of FcγR signalling on ILC3s. In particular, we hypothesised that FcγR cross-linking may regulate cytokine production and antigen presentation by these cells, given the canonical roles of FcγRs in regulating these processes across various innate immune cell populations.

To determine whether IgG ICs could promote IL-22 production, colonic inflamed LPMCs were stimulated with O-IC and IL-22 production measured by ELISA. In paired LPMC samples, IC stimulation consistently increased IL-22 production (Fig. 5.6A), while intracellular staining demonstrated an increased proportion of LPMCs expressing IL-22, with the majority of IL-22$^+$ cells being Lin$^-$. Flow cytometric identification of Lin$^-$ RORγt$^+$ IL-7Rα$^+$ ILC3s demonstrated an increased proportion of ILC3s expressing IL-22 and an increase in ILC3 IL-22 MFI in response to O-IC (Fig. 5.6B). However, there was minimal production of IL-17A production by ILC3s in the presence or absence of ICs.

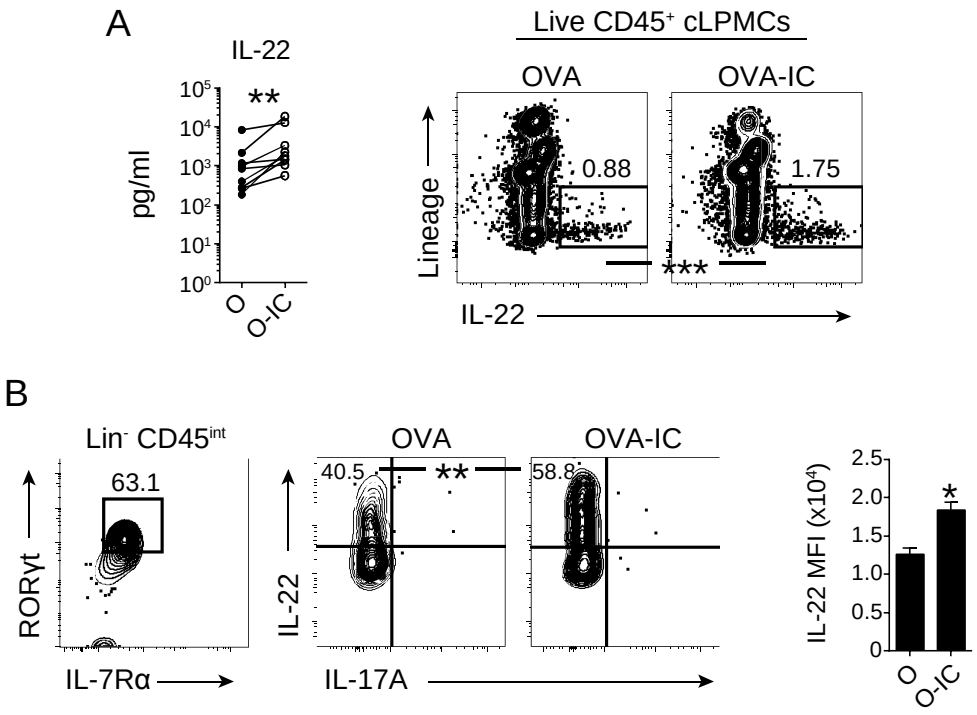

Figure 5.6. Immune complexes promote IL-22 production by ILC3s. (A) ELISA of IL-22 production by inflamed colonic LPMCs stimulated for 16 h with O or O-IC. (B) Intracellular cytokine staining of IL-22-producing cells following 6 h stimulation with O or O-IC. $n = 3$ per group. Data are representative of two independent experiments. *P* values were calculated using a ratio-paired *t* test (A) or a parametric Student's *t* test (B, C). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

ILC3s are known to express MHC class II molecules. In the GI tract, and specifically within GALT, ILC3s present antigen to T cells in the absence of CD80 and CD86, inducing T cell anergy [554], [587]. However, splenic ILC3s can activate T cell proliferation through MHC-II-dependent mechanisms [589]. MHC class II expression has been reported to be refractory to ILC3 stimuli. Strikingly, however, IC stimulation of LPMCs upregulated MHC-II expression by colonic ILC3s (Fig. 5.7A), as well as other cell-surface co-stimulatory molecules, including PD-L1 (Fig. 5.7B). Therefore, IgG IC can induce cytokine production and antigen presentation by ILC3s in mixed intestinal leukocyte populations.

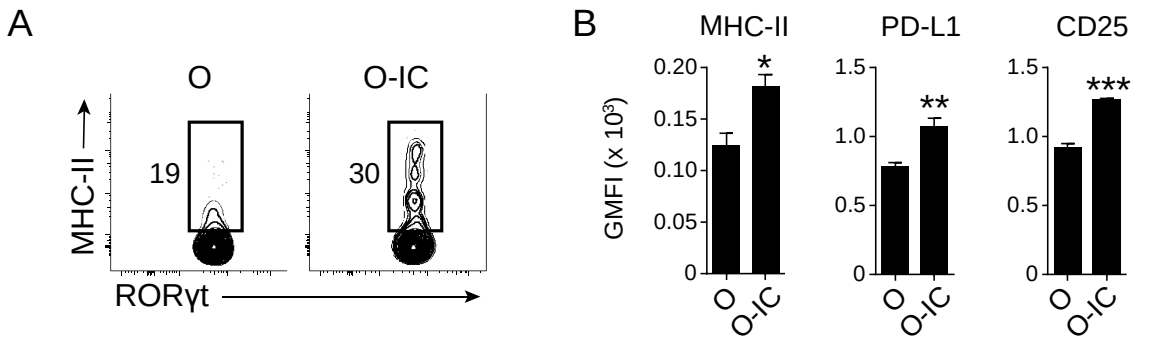

Figure 5.7. ICs induce ILC3 MHC-II upregulation. (A) MHC class II staining of RORγt$^+$ ILC3s in small intestinal LPMCs stimulated with O or O-IC for 16 h. *n* = 3 per group. (B) Quantification of cell surface receptor expression on ILC3s after LPMC stimulation. Data are representative of three independent experiments. *P* values were calculated using the parametric Student's *t* test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

In summary, stimulation of mixed colonic leukocyte cultures with IC induces the activation of ILC3s, both via induction of an IL-22-dominated cytokine response and the upregulation of MHC-II molecules. Therefore, IgG appears to favour a homeostatic programme in these cells that could reinforce barrier function and suppress T cell responses locally during inflammation. As such, IgG would be acting in a predominantly anti-inflammatory manner on these cells. However, the use of mixed cultures precludes investigation of the contribution of cell-intrinsic FcγR signalling towards these observations. To address this, we cross-linked FcγRs on flow-sorted ILC3s *in vitro*.

5.4. Analysis of canonical FcγR signalling in ILC3s

In order to dissect the effects of FcγR signalling on ILC3s, we first sought to determine the downstream signalling pathways involved. FcγR cross-linking leads to the activation of SYK-mediated signalling pathways. Therefore, we characterised phosphorylation of classical FcγR-associated signalling molecules. Indeed, 15 min FcγR cross-linking with 2.4G2 and a secondary IgG antibody resulted in enhanced phospho-SYK and phospho-ERK levels (Fig. 5.8).

Of note, FcγR cross-linking on flow-sorted ILC3s promoted their survival *in vitro*, although this effect was lost during longer incubation periods (Fig. 5.9).

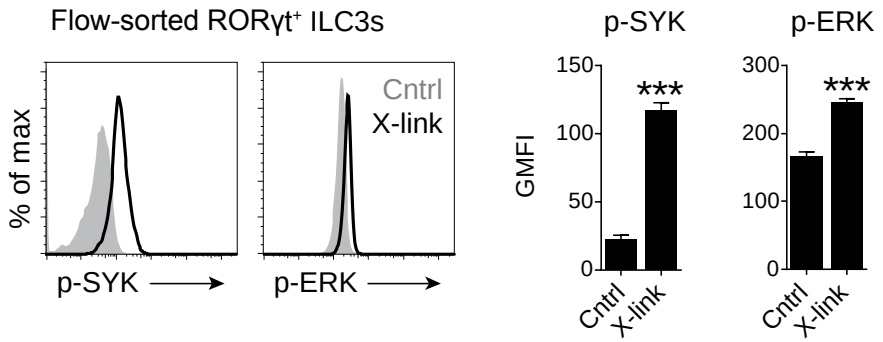

Figure 5.8. FcγR crosslinking activates canonical downstream FcγR signalling pathways. Intracellular phospho-staining and quantification of flow-sorted intestinal ILC3s following 15 min FcγR crosslinking with 2.4G2 and anti-rat IgG. Data are representative of two independent experiments. *P* values were calculated using a parametric Student's *t* test. *** $P < 0.001$.

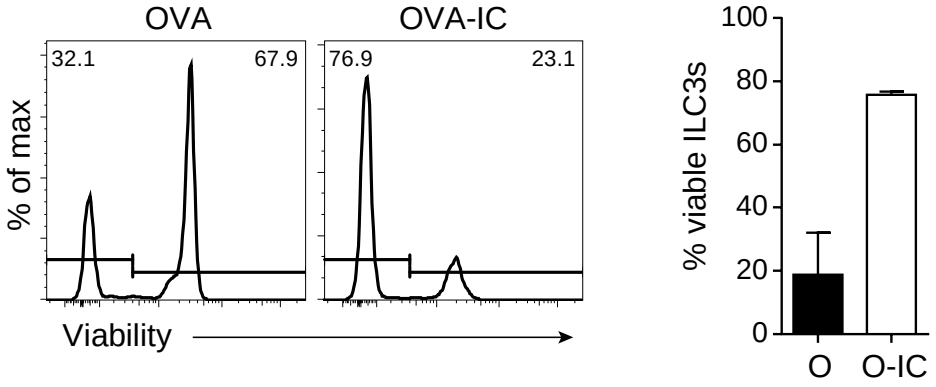

Figure 5.9. FcγR promotes ILC3 survival *in vitro*. Flow cytometric analysis of ILC3 viability *in vitro* after 16 h incubation in complete RPMI in the presence or absence of O or O-IC. Data are representative of three or more independent experiments.

5.5. The role of FcγR signalling on ILC3 antigen presentation

FcγRs are known to induce the phagocytosis of antigens by professional antigen-presenting cells and promote antigen presentation via MHC molecules. Therefore, we sought to determine whether IgG opsonisation increased antigen uptake and influenced MHC-II-mediated antigen presentation by ILC3s. Incubation of flow-sorted ILC3s with fluorescent OVA resulted in relatively low levels of antigen uptake, consistent with previously published data (Fig. 5.10) [543]. However, incubation with O-IC did not result in significant uptake, suggesting FcγR-mediated uptake is not a major facet of ILC biology. Indeed, NK cells are not documented to undergo FcγR-mediated phagocytosis.

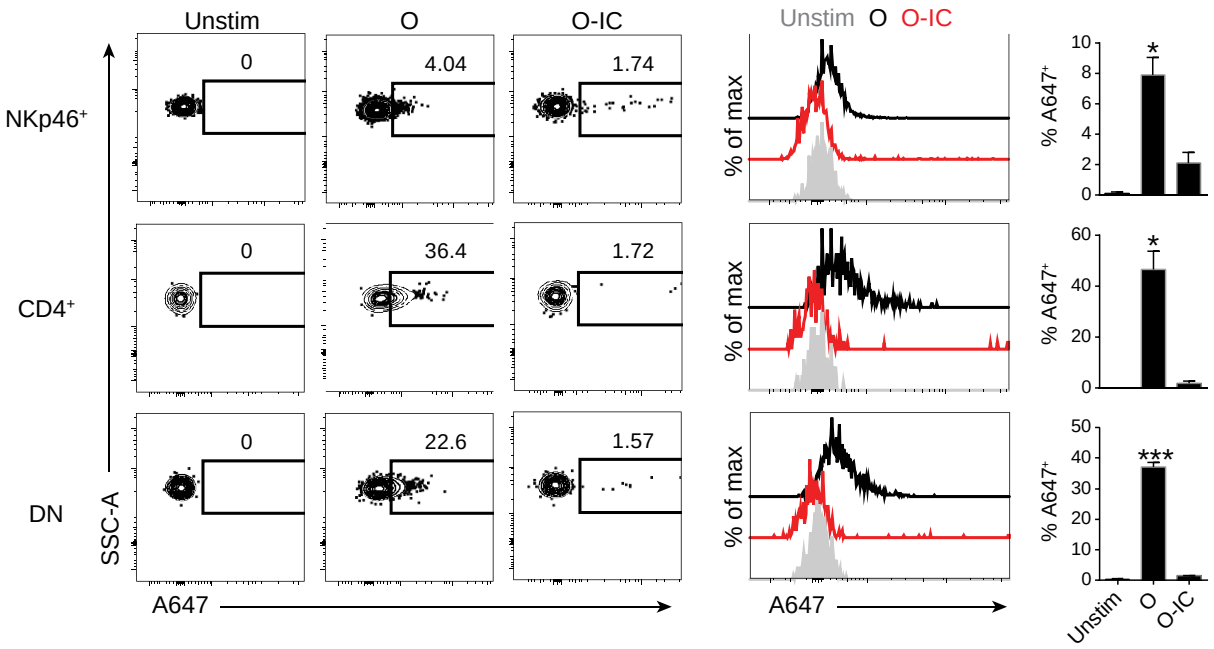

Figure 5.10. FcγR crosslinking does not enhance phagocytosis by ILC3s. Quantification of OVA-647 uptake by flow-sorted ILC3s following 16 h incubation with O-A647 or O-A647 immune complexes. *n* = 3 per group. Data are representative of three independent experiments. *P* values were calculated using the parametric Student's *t* test. * $P < 0.05$; *** $P < 0.001$.

As we had previously demonstrated MHC-II upregulation by ILC3s following IC stimulation of cLPMCs, we sought to investigate whether this was due to a direct effect of IC on ILC3s. Indeed, FcγR cross-linking with O-IC on flow-sorted intestinal ILC3s upregulated expression of MHC-II compared to O-stimulated controls, with little change in CD80 or CD86 expression, which remained negligible under all conditions tested (Fig. 5.11A). Furthermore, analysis of ILC3 subsets demonstrated that this effect was most pronounced for NKp46$^+$ ILC3s (Fig. 5.11B). While these cells have lower MHC-II expression than LTis, this may reflect their higher level of FcγRIII expression. Therefore, although O-IC does not promote antigen uptake by ILC3s, MHC class II expression is increased in response to FcγR ligation.

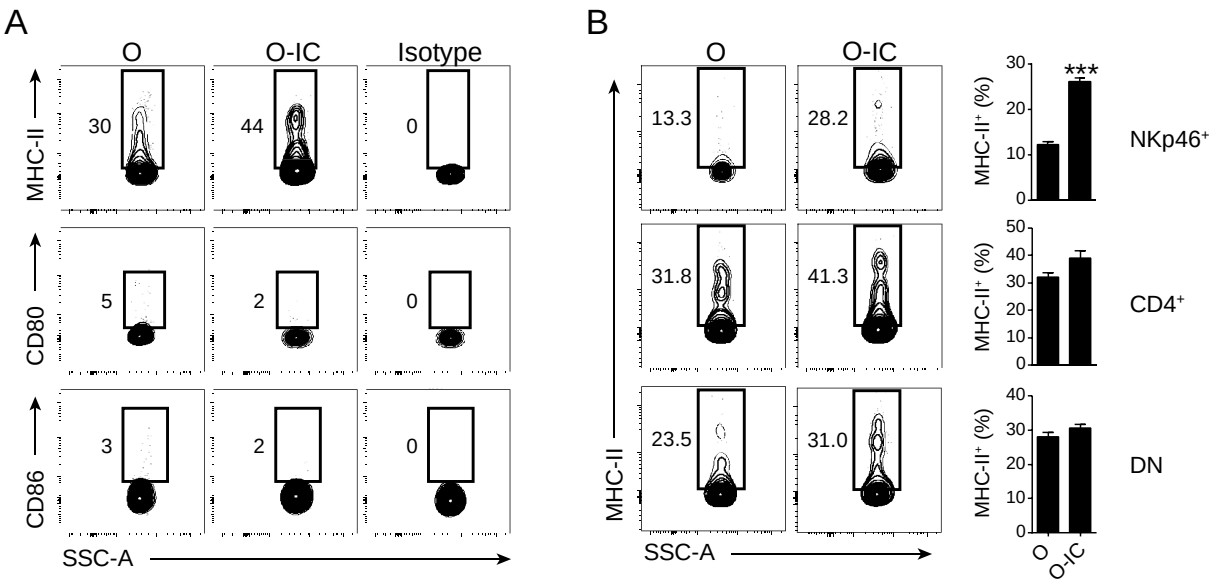

Figure 5.11. Immune complexes induce MHC class II expression by ILC3s. (A) Flow cytometry showing MHC-II and CD80/CD86 expression by RORγt$^+$ ILC3s following 16 h stimulation of small intestinal LPMCs with O or O-IC. (B) Stratification of RORγt$^+$ ILC3s into subsets and quantification of MHC class II upregulation in SI-LPMC cultures following 16h stimulation with O or O-IC. n = 3 per group. Data are representative of three independent experiments. P values were calculated using the parametric Student's t test. *** $P < 0.001$.

5.6. Modulation of ILC3 FcγR expression during inflammation

FcγR expression is modified by local inflammation in intestinal macrophages, with an increase in A/I ratio driven by upregulated expression of activating receptors. We therefore hypothesised that inflammation may also influence ILC3 FcγR expression.

Flow-sorted intestinal ILC3s from inflamed mice demonstrated an increased expression of *Fcgr2b* transcripts relative to ILC3s isolated from healthy mice (Fig. 5.12A). Furthermore, *Fcgr3* and *Fcer1g* expression levels were reduced in SI-resident ILC3s, and relatively unchanged in colonic ILC3s, isolated from the inflamed GI tract compared to uninflamed controls. This demonstrates a reduction in the A/I ratio following the induction of inflammation, the opposite to what is seen in intestinal macrophages, and is driven by an upregulation in FcγRIIB expression.

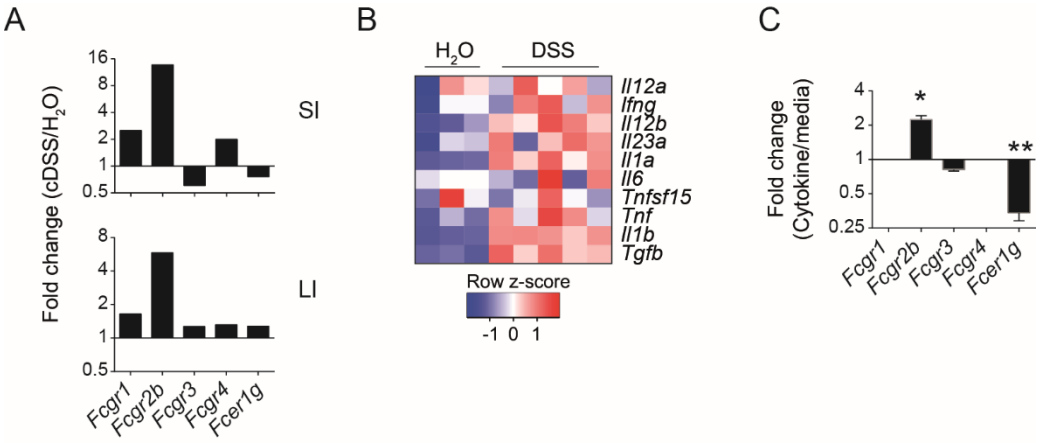

Figure 5.12. An inflammatory milieu induces ILC expression of the inhibitory FcγRIIB. (A) qPCR analysis showing fold change in FcγR expression in SI and colon-resident ILC3s flow-sorted from DSS-inflamed mice relative to healthy controls. (B) Heatmap showing inflammatory cytokines in DSS-inflamed whole colon tissue compared to healthy control tissue (GSE42768). (C) qPCR analysis showing fold change in FcγR gene expression by flow-sorted intestinal ILC3s following 24 h incubation with IFN-γ, TNF and IL-1β relative to untreated ILC3s. n = 3 per group. Data represent a single experiment. P values were calculated using the parametric Student's t test. * $P < 0.05$; ** $P < 0.01$.

DSS is associated with the induction of a broad local inflammatory response. We sought to identify key DSS-associated cytokines that may play a role in modulating FcγR expression in ILC3s through analysis of transcriptomics data (Fig. 5.12B). As well as increased levels of IL-1β, DSS-induced colitis also increased expression of several ILC3-activating cytokines, including IFN-γ and IL-23. Culture of flow-sorted intestinal ILC3s from uninflamed mice with a cocktail of inflammatory cytokines (IL-1β, IFN-γ, TL1A) resulted in significantly increased expression of *Fcgr2b*, a reduction in *Fcer1g* levels, and a minor reduction in *Fcgr3*, consistent with *in vivo* observations (Fig. 5.12C).

5.7. ILC3 activation in *Fcgr2b*-deficient mice

Anti-commensal IgG and commensal opsonisation is significantly induced during chronic inflammatory disorders of the GI tract, as demonstrated in Chapter 3. Therefore, it is likely that FcγR ligation on intestinal ILC3s becomes most prominent under these circumstances. Therefore, we sought to investigate whether local IgG induction in chronic intestinal inflammation may influence ILC3 phenotype and function. Firstly, we characterised IL-22 and IL-17A-producing cell subsets in chronic DSS-induced colitis. Chronically inflamed colonic LPMCs produced increased levels of IL-22 and IL-17A in culture compared to uninflamed cells (Fig. 5.13A). Flow cytometry identified that, although both IL-17A and IL-22 production was confined to the CD90.2$^+$ population (Fig. 5.13B), the majority of IL-22-producing CD90.2$^+$

lymphocytes were CD3ε^{-} CD4$^{+/-}$, consistent with ILCs, while IL-17A production was predominantly from CD3ε^{+} CD4$^{+/-}$ cells, consistent with T cells (Fig. 5.13C).

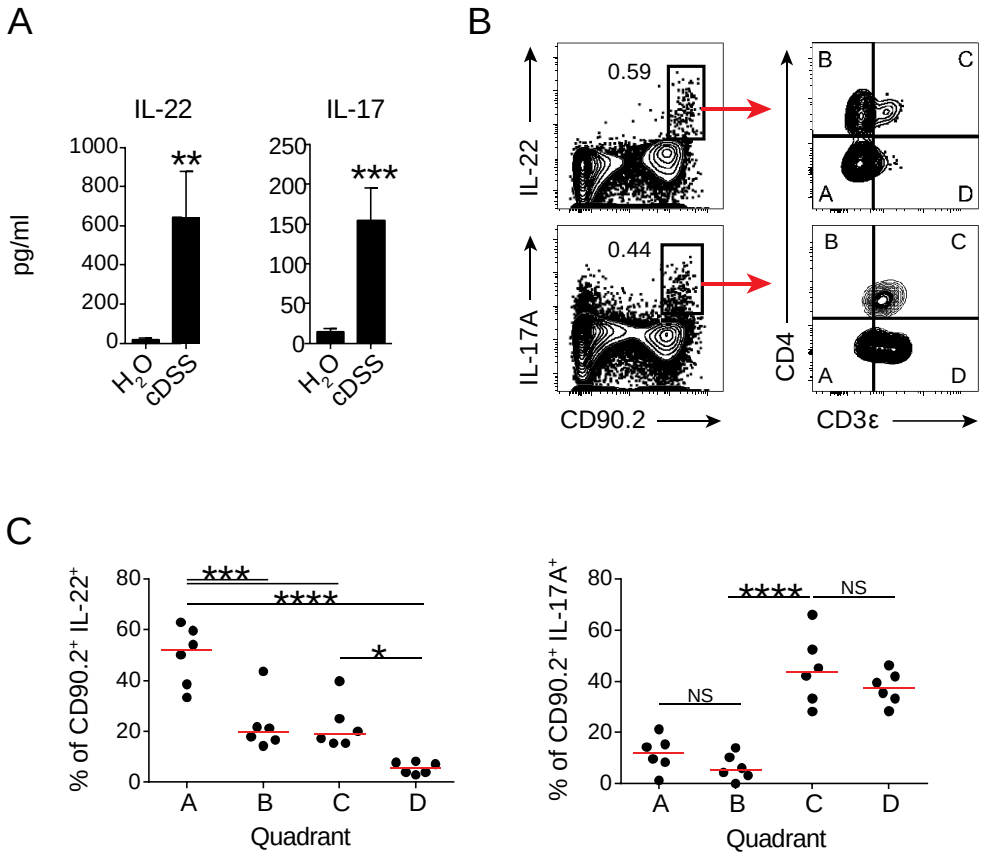

Figure 5.13. Group 3 innate lymphoid cells are the major source of IL-22 in chronic intestinal inflammation. (A) ELISA of IL-22 and IL-17A production by colonic LPMCs per 8 x 10^5 cells. *n* = 5 per group. (B) Flow cytometry staining of IL-22 and IL-17A producing cells in the inflamed colonic lamina propria following chronic DSS administration. (C) Quantification of the cytokine producing subsets shown in B. Data are representative of two or three independent experiments. *P* values were calculated using the parametric Student's *t* test (A) or a one-way ANOVA (C). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Analysis of IL-22 production in *Fcgr2b*-deficient mouse colons following cDSS colitis demonstrated a significant increase in intestinal CD90.2^{+} IL-22^{+} ILC3s in the absence of FcγRIIB (Fig. 5.14A). This increase was independent of IL-1 signalling, as IL-1R1 blockade had no effect on IL-22 production (Fig. 5.14A, B). CD4^{-} ILC3s were more abundant than CD4^{+} ILC3s, but both populations were similarly enriched in inflamed colons of *Fcgr2b*-deficient mice (Fig. 5.14B).

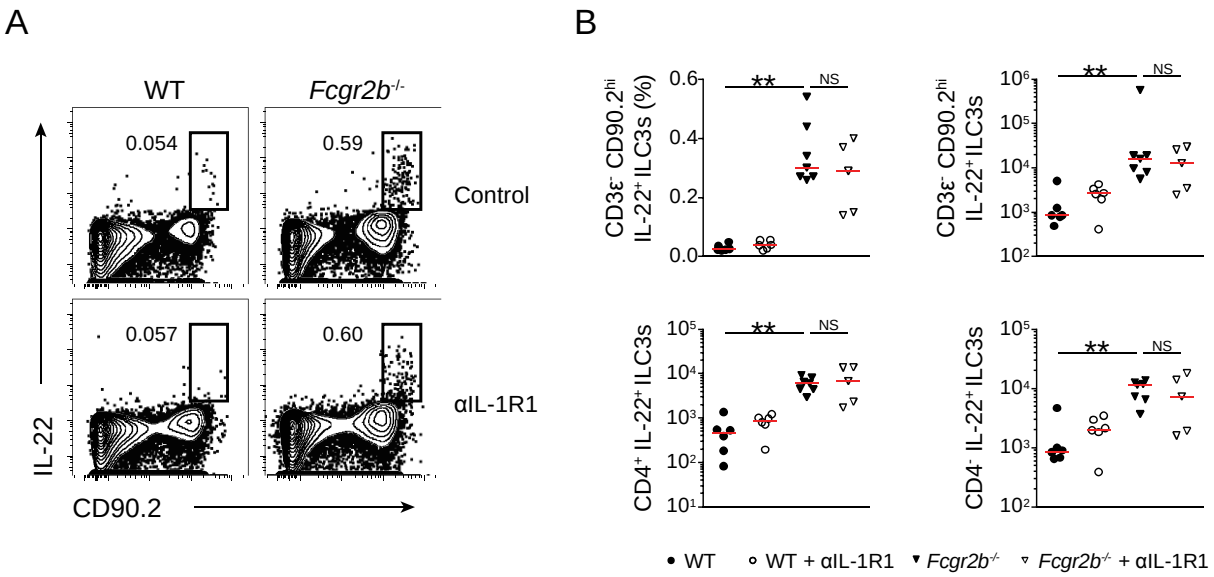

Figure 5.14. IL-22-producing ILCs are enriched in inflamed colons from *Fcgr2b*-deficient mice. (A) Flow cytometry staining of IL-22-producing CD3ϵ$^-$ CD90.2$^+$ cells in the inflamed colons of WT and *Fcgr2b*-deficient mice treated with control IgG or anti-IL-1R1 IgG. (B) Quantification of IL-22$^+$ ILC frequency and subset numbers in mice shown in A. Data are representative of two independent experiments. *P* values were calculated using the nonparametric Mann-Whitney test. ** $P < 0.01$.

In summary, enhanced Th17 responses are characteristic of chronic intestinal inflammation, with ILC3s being the major source of IL-22 within the inflamed colonic mucosa. We observed little IL-17A secretion by ILC3s, and was largely confined to the CD3ϵ$^+$ T cell compartment, supportive of studies in Chapter 4. In *Fcgr2b*-deficient mice, IL-22 production by CD3ϵ$^-$ CD90.2^{hi} ILC3s was significantly increased relative to WT controls, and this was refractory to IL-1R1 blockade. This is in stark contrast to observations made in Chapter 4 that IL-17A and IL-22 production by colonic T cells was heavily dependent on IL-1 signalling. These results reinforce the suggestion that IgG IC supports an IL-22-mediated homeostatic response in ILC3s, with little inflammatory IL-17A, and demonstrates IL-1β-independent mechanisms for ILC3 activation in response to IgG, although this may be through several mechanisms.

5.8. Global transcriptional profiling of IgG-stimulated ILC3s

To get a better understanding of how FcγR signalling may contribute to ILC3 biology, we performed RNAseq analysis on flow-sorted small intestinal ILC3s stimulated for 4 h with O or O-IC. Surprisingly, GSEA of Hallmarks pathways demonstrated a downregulation of several pathways following IC stimulation, as opposed to what is seen in IC-stimulated macrophages (Fig. 5.15A). In particular, notable reductions were seen in *TNFA signalling via NFκB*, *MTOR complex 1 (MTORC1) signalling*, *Glycolysis*, *Hypoxia*, and *Cholesterol homeostasis*, modules classically associated with the inflammatory response (Fig. 5.15B).

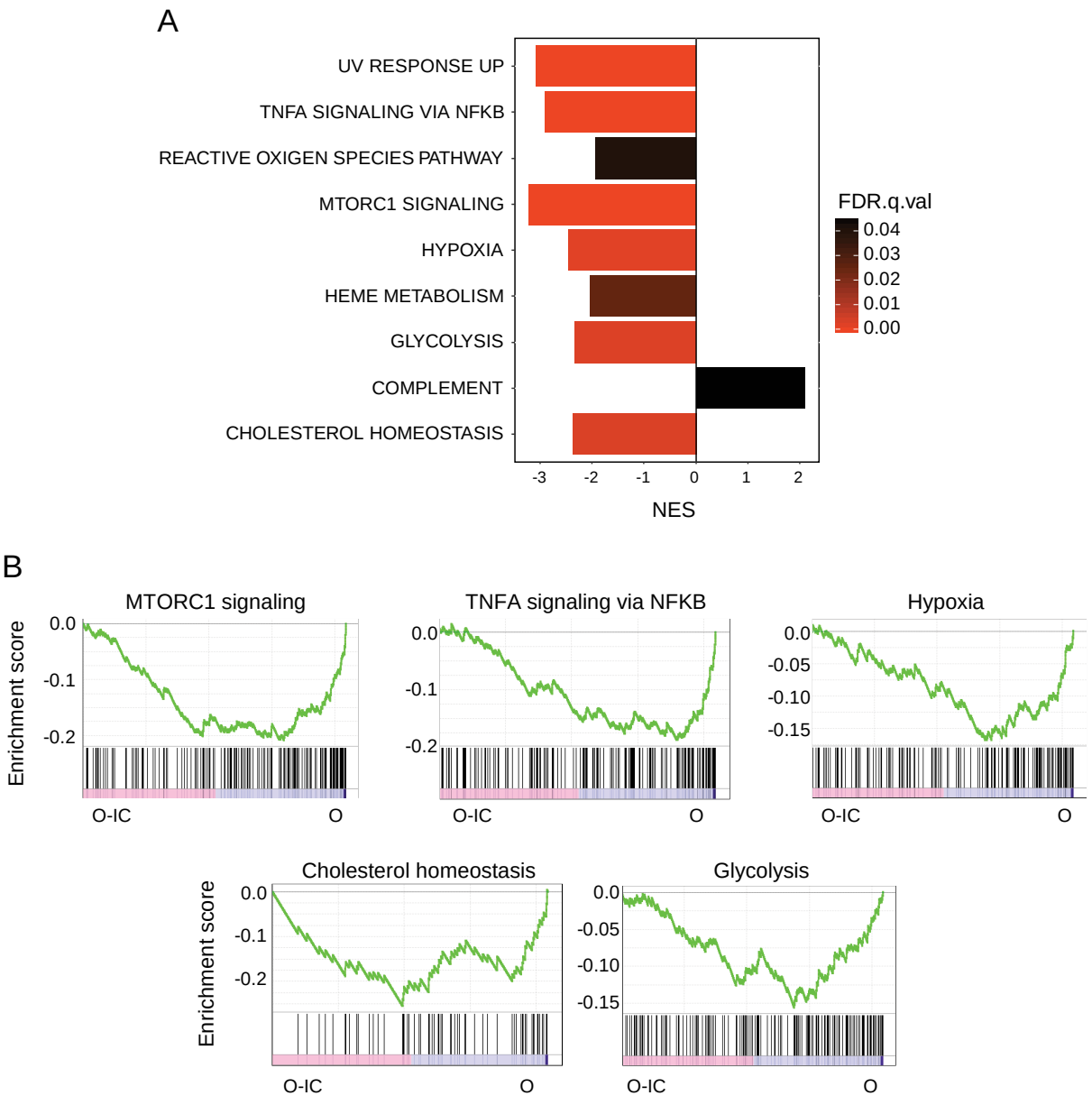

Figure 5.15. Transcriptomics analysis of ILC3s reveals an anti-inflammatory profile induced by FcγR signalling. GSEA of Hallmarks pathways in IC-stimulated flow-sorted intestinal ILC3s compared to O-stimulated cells.

Analysis of the top differentially expressed genes in response to IC stimulation demonstrated a reduction in several activation-associated molecules, including *Stat1*, *Csf2*, *Il2ra*, *Ptgs2* and *Cd69* (Fig. 5.16A, B). *Csf2* encodes GM-CSF, which has been implicated in driving detrimental chronic colitis through the local activation of granulocytes. Therefore, IC-stimulated ILC3s may downregulate inflammatory responses predominated by GM-CSF secretion in favour of a transcriptional programme supporting barrier function and T cell inactivation. This would represent a novel negative feedback loop to regulate type 17 responses during chronic inflammation. Supporting this, IC-induced genes included those associated with the reinforcement of the ILC3 phenotype, such as *Il7r*, *Zfp292*, and *Ikzf3* (gene encoding Aiolos), as well as certain NK-associated genes not previously associated with ILC3 function, including *Cd226* (Fig. 5.16A, C).

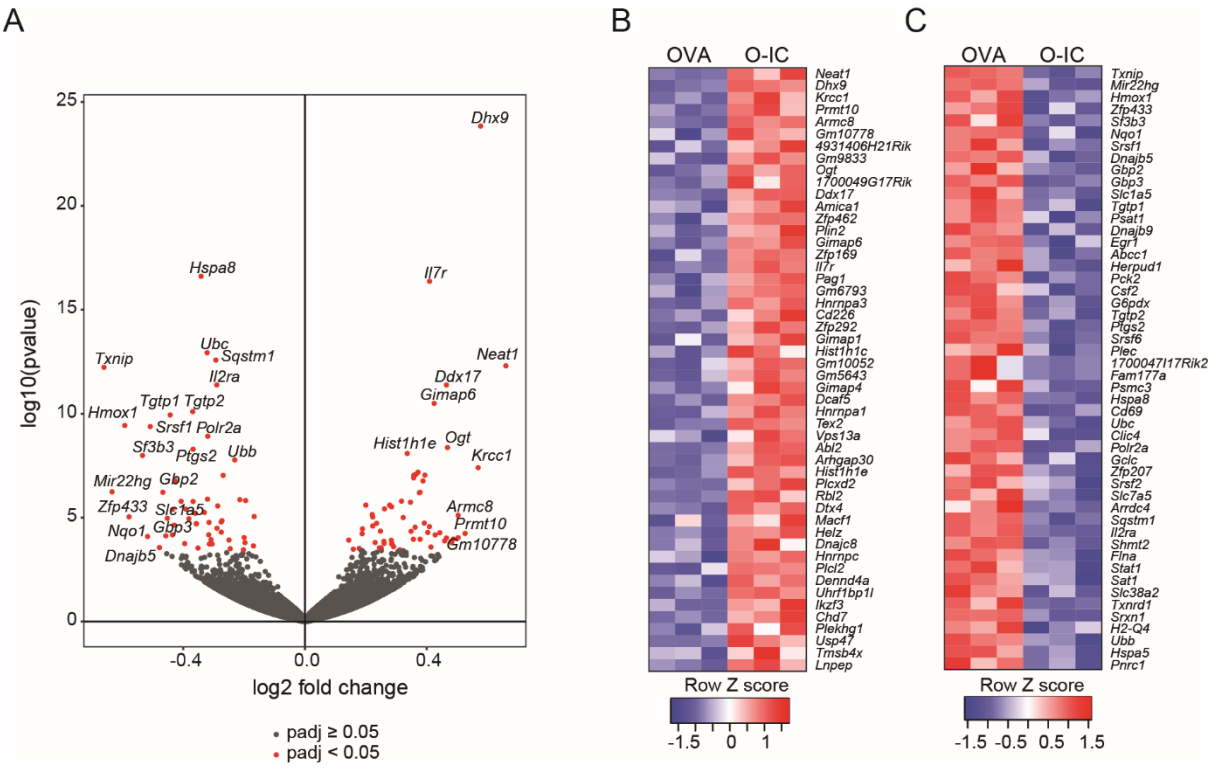

Figure 5.16. **Analysis of top differentially expressed genes in response to IC.** (A) Volcano plot showing to differentially expressed genes in response to IC stimulation, as a function of *P* value and log2 fold change. (B, C) Heatmaps showing the top 50 upregulated (B) and the top 50 downregulated (C) genes in response to IC stimulation.

Network analysis was performed by STRING analysis on the top 50 up- and down-regulated genes (Fig. 5.17). Amongst downregulated networks were genes associated with the spliceosome and amino acid transporters, as well as, most notably, a cluster of genes associated with *Stat1* signalling, as previously mentioned. IC stimulation induced pathways involved in the regulation of transcription and translation: upregulated networks included genes

involved in RNA processing, GTPase activity, and IL-7R maintenance. IC-induced gene expression changes were smaller than those in macrophages, and likely reflects the lower relative expression of FcγRs compared to the latter.

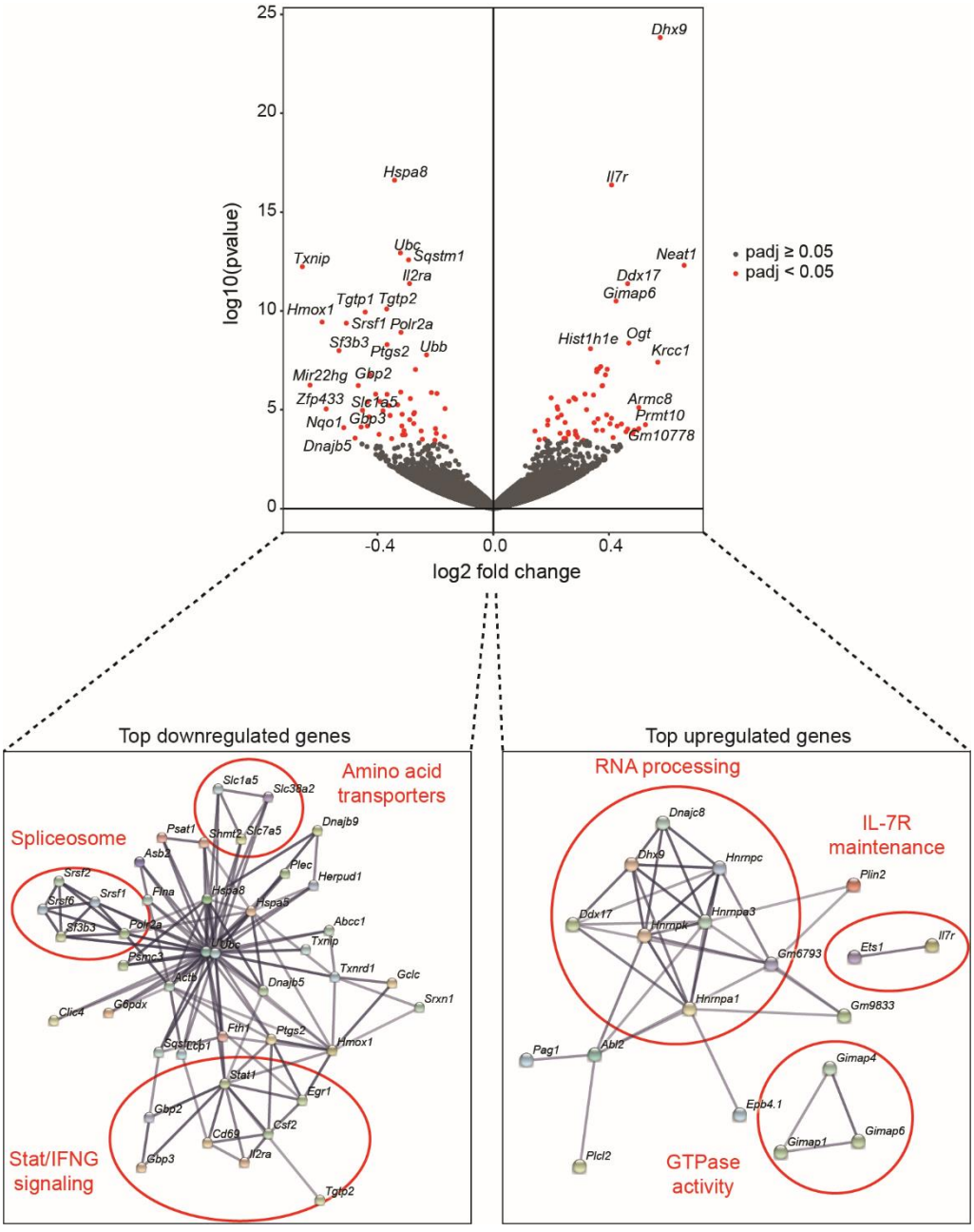

Figure 5.17. STRING analysis reveals regulation of genes involved in RNA processing and Stat signalling.

Given the largescale changes in networks associated with RNA processing, we analysed the differential regulation of transcriptional and translational regulators in response to IC stimulation (Fig. 5.18). Strikingly, IC-induced genes included *Zfp292*, *Zbtb16* (gene encoding PLZF), *Tbx21* (gene encoding T-bet), and *Ikzf3* (gene encoding Aiolos, an epigenetic modifier associated with the maintenance of Th17 cells), several genes associated with ILC3 development, maintenance, and function. These observations support the notion that FcγR signalling is beneficial for the maintenance of ILC3s within the GI tract. Therefore, we sought to further investigate transcriptional programmes associated with ILC3 biology within the GI tract: SI-associated genes, ILC3-associated cytokine and chemokine receptors, and TF pathways.

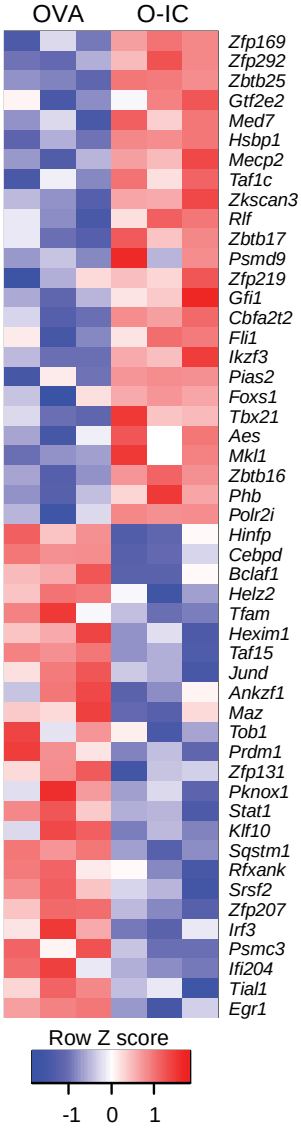

Figure 5.18. FcγR signalling modulates expression of transcriptional regulators involved in ILC identity. Heatmap showing differentially expressed transcriptional regulators in response to IC stimulation.

RNAseq analysis of ILCs has previously demonstrated that SI-resident ILCs are associated with a unique transcriptional signature distinct from ILCs in distal tissues, such as the liver [556]. We found that IC stimulation induced widespread changes in this SI-associated signature (Fig. 5.19A), suggesting that tissue-residency influences the response of cells to FcγR ligation.

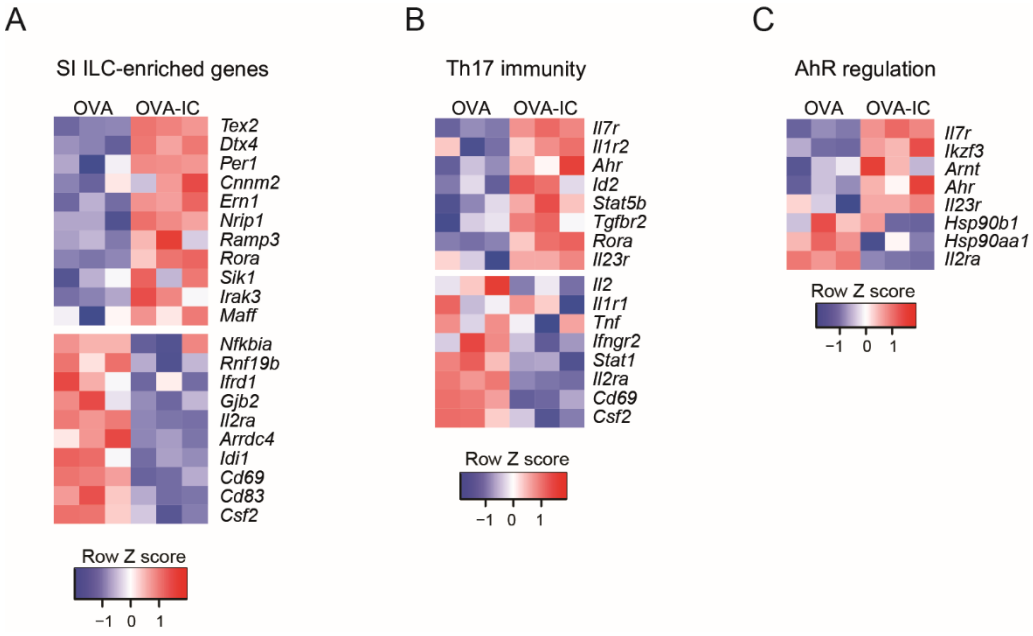

Figure 5.19. FcγR signalling induces transcriptional profiles associated with tissue-residency and ILC3 maintenance. Heatmaps showing differential gene expression of genes associated with SI ILC biology (A), maintenance of Th17 immunity (B), and the regulation of AhR signalling in ILC3s in response to IC.

Recent data has demonstrated ILC subset plasticity within tissues in response to local cues: IL-2, IL-12, and IL-18 are associated with an ILC3 to ILC1 transition, driven by STAT5 signalling, while IL-1β, IL-23, and IL-7 promote the maintenance of ILC3s [601]–[608]. We therefore sought to determine the role of IC on genes associated with ILC3 maintenance and function within the GI tract. As well as the previously highlighted genes, we also identified several other genes associated with reinforcement of the ILC3 phenotype and inhibition of the ILC1 transition (Fig. 5.19B): *Ahr*, *Id2*, *Rora*, *Il23r*, and *Tgfbr2*. AHR was of particular interest. It is essential for ILC3 maintenance and cytokine production within the GI tract, driven by responses to endogenous and exogenous aryl hydrocarbon ligands [592], [594], [609]. AHR is maintained in the cytoplasm through interaction with Hsp90. We observed a decrease in Hsp90 subunit expression in response to O-IC (Fig. 5.19C). Upon ligand binding, AHR associates with ARNT to form a functional TF. Strikingly, *Arnt* was similarly induced by O-IC, demonstrating that FcγR signalling regulates several component of the AHR transcriptional machinery, potentially driving elevated IL-22 production *in vivo*.

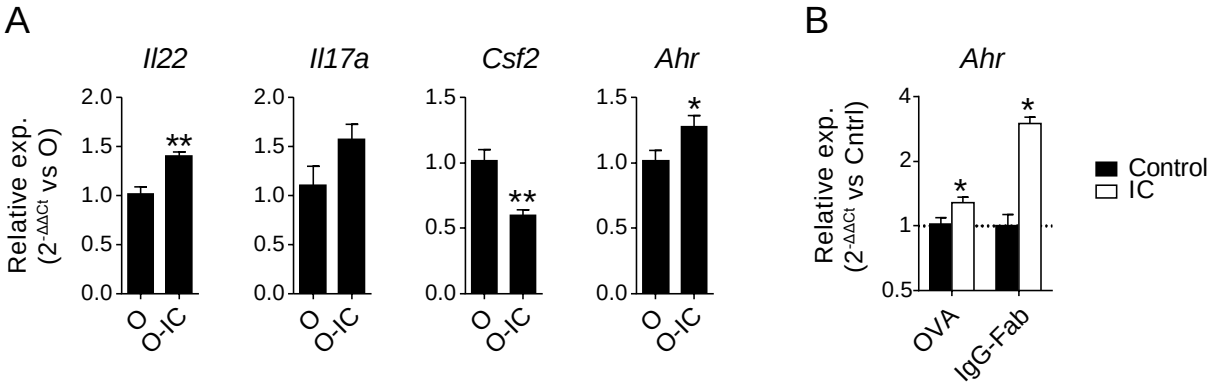

Figure 5.20. IC stimulation regulates ILC3 cytokine production and AhR expression. (A) qPCR of ILC3-associated cytokine genes and *Ahr* in flow-sorted ILC3s stimulated with IC. *n* = 6 per group. (B) qPCR analysis of *Ahr* expression in flow-sorted ILC3s stimulated with different types of IC, insoluble OVA-ICs and soluble IgG-F'ab ICs. *n* = 3 per group. Data are representative of two or three independent experiments. *P* values were calculated using the parametric Student's *t* test. * $P < 0.05$; ** $P < 0.01$.

qPCR was used to confirm the effects of IC on flow-sorted ILC3s. Interestingly, while non-significantly increased in the RNAseq dataset, *Il22* expression was significantly increased by qPCR in response to IC (Fig. 5.20A), perhaps due to the increased number of technical replicates. Reduced *Csf2* expression and increased *Ahr* expression was also demonstrated (Fig. 5.20A). Furthermore, *Ahr* expression was increased in response to stimulation with different forms of IC – insoluble OVA-anti-OVA IgG ICs and soluble murine IgG-Fab-anti-IgG ICs (Fig. 5.20B).

Given these changes in core ILC-associated genes, we hypothesise that FcγR signalling promotes the maintenance of ILC3s within tissues via the induction of transcriptional regulators, such as AHR, RORα, PLZF, T-bet, epigenetic modifiers, such as Aiolos, and cytokine and chemokine receptors, including IL-23R and CCR9, while simultaneously reducing response to ILC1-inducing factors, such as IL-2, via downregulation of IL-2Rα and STAT1/5 signalling pathways (summarised in Fig. 5.21).

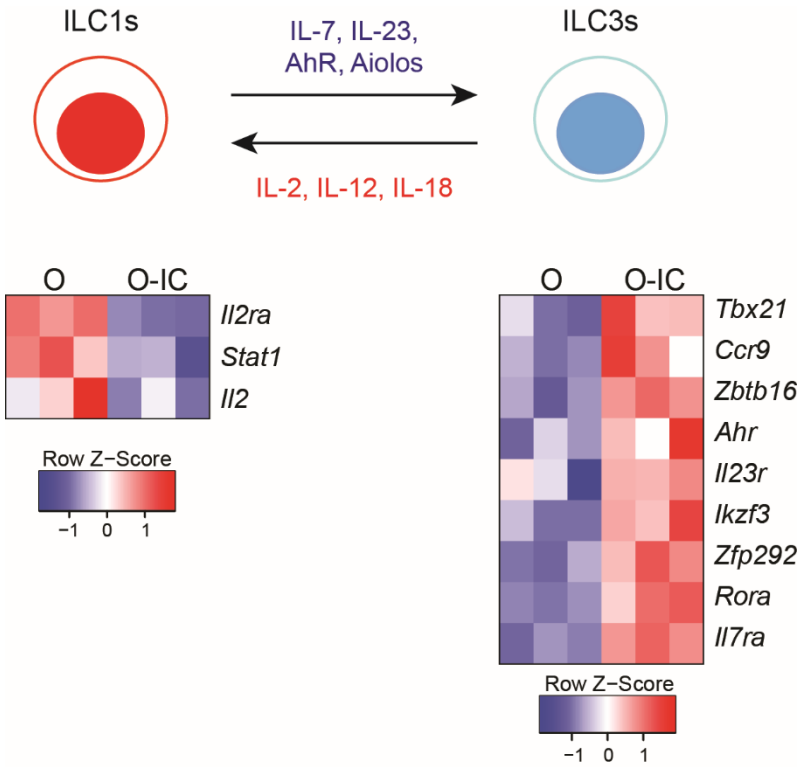

Figure 5.21. Transcriptional changes associated with ILC3 maintenance and tissue residency. Schematic showing the regulation of ILC1-to-ILC3 plasticity (above) and IC-induces transcriptional changes (below) of genes associated with plasticity.

5.9. Discussion

5.9.1. ILC1s and ILC3s express FcγRs

ILCs are a recently identified class of immune cells that are enriched at mucosal sites, such as the GI tract [528]. Derived from the CLP, ILCs represent a distinct group of innate lymphocytes characterised by the absence of somatically-recombined receptors and the potent, rapid production of inflammatory cytokines in response to local inflammatory mediators. Three major ILC subsets exist, ILC1s, ILC2s and ILC3s, with ILC3s being particularly enriched in the GI tract. Here, they have important roles in the maintenance of the epithelial barrier and activation of local haematopoietic and non-haematopoietic immune cells through production of IL-22, IL-17A and GM-CSF. These cells are critically dependent on other tissue-resident immune cells for activation, as well as the epithelium, with MNP-derived IL-23, IL-1β and TL1A implicated in ILC3 cytokine production in several models of murine colitis [292], [361], [399]. T-bet^{+} NKp46^{+} ILC3s are predominantly located in the LP in close proximity to the epithelium [580], while so-called LTi CCR6^{+} CD4$^{+/-}$ ILC3s are located within the GALT, where they regulate SLO development and T cell activation by production of Ltα1β2 and MHC-II-mediated antigen presentation, respectively [546], [554], [587]. In contrast, IFNγ-producing ILC1s are particularly enriched during colitis, thought to be driven by IL-12-mediated plasticity from local ILC3s and ILC2s [597], [601].

In this chapter, we set out to profile the expression pattern of FcγRs across ILC subsets within the GI tract. We demonstrated that T-bet^{+} NKp46^{+} ILC1s and all subsets of ILC3s expressed FcγRs, while ILC2s demonstrated negligible expression. FcγRIII was the most highly expressed, consistent with NK cell expression patterns, with FcγRIIB upregulated in the context of inflammation. FcγR expression was common to ILC1s and ILC3s across several tissues, including extra-GI tissues like the spleen.

The results presented here highlight two important facets of ILC biology. Most notably, there is a clear divide between type 1/17 responses and type 2 responses in their requirement to sense IgG. Type 2 responses are typically associated with allergy and the induction of an IgE response. Therefore, under conditions of IgE-dominated humoral responses in which ILC2s are activated, expression of receptors for IgG may seem unnecessary. It is curious, however, that we did not observe significant FcεR expression by ILC2s in Immgen transcriptomics datasets (data not shown).

Secondly, highest FcγR expression coincided with co-expression of T-bet. T-bet is required for NK cell function and, as such, may contribute to FcγR expression by these subsets directly. However, profiling of TF binding sites in FcγR genes has not been carried out here. More importantly, previous studies have highlighted a plastic relationship between ILC3s and ILC1s, dictated by environmental stimuli and the regulation of RORγt and T-bet expression [356],

[357], [547], [600], [601]. ILC3s can develop in ILC1-like cells under the influence of IL-2 and IL-12, giving rise to the possibility that FcγR-expressing ILC1s could arise from the transition of FcγR-expressing ILC3s and vice versa. Furthermore, FcγR ligation could play a direct role in regulating this plasticity to suit the needs of the immune response. For example, FcγR signalling in ILC3s may promote a transition to ILC1s to support IFNγ-mediated inflammation. Alternatively, FcγR signalling in ILC3s may suppress transition to ILC1s to drive IL-22-mediated reinforcement of the epithelium during colitis. Given the differential association of ILC1s and ILC3s with inflammation, this would have important consequences for local inflammatory responses.

5.9.2. Immune complexes promote ILC3 activation *in vitro*

In support of an ILC3-promoting effect, we observed an increase in IL-22 and MHC-II expression by ILC3s following LPMC stimulation with IgG IC. Minimal IL-17A production by ILC3s was observed and was not influenced by stimulation condition.

The use of mixed LPMC cultures will result in confounding effects by IC-inducible cytokines secreted by other cells, for example macrophages. Indeed, other than the IL-1β-dominated response highlighted in Chapter 4, IC signalling has also been shown to promote production of IL-23, PGE2, TNF, and TL1A, all of whom have been shown to regulate ILC3 cytokine production [291], [360], [544], [570]. These effects may overwhelm any effect of ILC3-intrinsic FcγR signalling. Further studies blocking IC-inducible cytokines individually or in combination would be required to resolve this. However, it remains possible that ILC3-intrinsic FcγR signalling cooperates with FcγR signalling on other GI-resident immune cells to induce a network that supports IL-22 production. For example, IC signalling may promote expression of IL-1 or IL-23 receptors or co-factors that promote ILC3 responsiveness to stimulation. Therefore, these results are important in demonstrating that IgG can regulate ILC3 biology, at least *in vitro*. Importantly, MHC-II expression by GI-resident ILC3s was previously demonstrated to be refractory to cytokine stimulation [554]. As such, it is possible that this effect is predominated by FcγR signalling intrinsic to ILC3s.

Unfortunately, no analysis of IFNγ or GM-CSF secretion by IC-stimulated LPMCs was carried out. Could FcγR signalling would suppress IFNγ secretion by ILC1s? Doping of LPMC cultures with congenic ILC3s would allow for analysis of ILC3-to-ILC1 transition in response to FcγR stimulation: if ICs regulate ILC plasticity, one might observe a decrease in the emergence of ILC1-like cells in cultures in response to IC. Exogenous cytokines known to influence ILC plasticity could also be added to monitor the effect of FcγR on these transitions.

5.9.3. FcγR signalling induces MHC-II expression but not antigen phagocytosis

We observed an increase in MHC-II expression on flow-sorted ILC3s in response to IC stimulation, supporting the hypothesis that this upregulation was intrinsically regulated by IgG. MHC-II induction occurred in the absence of changes to CD80/86, which remained minimal. These results demonstrate that IC stimulation promotes an anti-inflammatory phenotype associated with the induction of T cell anergy and apoptosis. Splenic ILC3s, however, are known to express CD80 and CD86, which can be upregulated in response to cytokines, such as IL-1β [589]. FcγR signalling on DCs is known to induce similar changes in MHC-II and CD80 and CD86, raising the possibility that FcγR cross-linking on splenic ILC3s promote T cell activation, rather than apoptosis.

The contribution of MHC-II-mediated antigen presentation *in vivo* remains unclear. In line with other studies [543], we observed a relatively minor capacity for antigen internalisation compared to professional APCs. However, unopsonised antigen uptake was most notable in LTi cells, which exhibit highest level of MHC-II expression. As these ILC3s were flow-sorted from the SI, it may be that GALT-resident ILC3s are more specialised for antigen presentation. It is notable, however, that we observed the most pronounced upregulation of MHC-II molecules by the NKp46^{+} ILC3 subset: these cells had the lowest MHC-II expression at rest, and are known for their residence within the LP itself, rather than the GALT. Furthermore, these cells expressed the highest levels of FcγRs. Whether significant antigen presentation may occur within the LP itself, however, is unclear, given the absence of structured lymphoid architecture. Therefore, the functional significance of these observations in not certain.

In these studies, we used O-IC, a model of insoluble ICs. It may be that ILC3s are more specialised for the uptake of smaller soluble ICs, consistent with their ability to promote antigen presentation of internalised peptides, rather than the efficient processing of large proteins. Alternatively, FcγR signalling may promote antigen presentation of smaller acquired peptides independently of opsonisation, such that FcγR signalling cooperates with antigen presentation but is functionally uncoupled from it. This would represent a novel function of FcγR signalling on innate APCs, but remains to be fully explored here.

5.9.4. ILC3 FcγRIIB expression is upregulated during inflammation

Inflammatory cytokines are known to alter the FcγR A/I ratio of several immune cells, such as macrophages and DCs [84]. Indeed, we demonstrated in Chapter 4 that macrophages within the GI tract increase their A/I ratio during colitis driven by upregulation of activating FcγR expression. Here, we demonstrated upregulation of the inhibitory FcγRIIB on ILC3s following chronic DSS-induced colitis, relative to controls. This effect could be recapitulated *in vitro*

through the incubation of flow-sorted ILC3s with a cocktail of inflammatory cytokines, including IFNγ.

We have hypothesised throughout this chapter that FcγR signalling promotes the reinforcement of the ILC3 phenotype, with elevated IL-22 production and MHC-II expression in response to IgG IC. These results raise the possibility that during inflammatory responses, upregulation of FcγRIIB actively inhibits this reinforcement, reducing the effect of activating FcγR signalling and promoting a transition to an ILC1-like phenotype consistent with the emergence of ILC1s during inflammation [597]. In this setting, FcγRIIB would be functionally opposed to its canonical role in the suppression of inflammation. In support of this, *Fcgr2b*-deficient mice had elevated IL-22-producing ILC3 numbers that was independent of IL-1 signalling. Given the role of FcγRIIB in the suppression of type 17-inducing cytokine production by MNPs, it is likely that FcγR signalling contributes indirectly to this elevated cytokine production, as stated previously for LPMC stimulations. *In vitro* studies with WT and *Fcgr2b*-deficient ILC3s isolated from inflamed mice would help to determine the contribution of this inhibitory receptor to ILC function in this context. Gating on IL-22-producing ILC3s did not allow for discrimination between a change in the total number of ILC3s between WT and *Fcgr2b*-deficient mice or a change in IL-22 production by the same number of total ILC3s. An increase in the total number of ILC3s regardless of IL-22 production may reflect additional developmental/homeostatic programmes that are influenced by FcγR signalling, for example migration to the GI tract or survival within tissues. In contrast, specific effects on cytokine production in the absence of FcγRIIB may more directly reflect a role for the inhibitory receptor in regulating the activation state of ILC3s, either directly or indirectly.

Regardless of intrinsic or extrinsic effects of FcγRIIB, the increased IL-22 production in KO mice raises numerous potential consequences for epithelial barrier function. Whether intestinal epithelial cells exhibit elevated STAT3 signalling and proliferation, enhanced mucus production, or crypt regeneration are all areas of ongoing interest. Indeed, we previously observed an increase in Reg family anti-microbial peptide production in *Fcgr2b*-deficient colons following chronic DSS-induced colitis, a facet known to be dependent on IL-22. Whether these effects would translate into secondary resistance to challenge by enteropathogens, such as *C. rodentium*, would be of great interest.

5.9.5. Global transcriptional profiling of IgG-stimulated ILC3s

RNAseq analysis of flow-sorted ILC3s stimulated with O-IC yielded global insights into the role of FcγR signalling in these cells. Strikingly, IC downregulated several inflammation-associated Hallmarks pathways, including TNFA signalling, MTORC1 signalling, and glycolysis. Furthermore, STRING analysis demonstrated significant downregulation of genes associated with STAT1 signalling and GM-CSF production in favour of RNA processing machinery.

Unlike in intestinal macrophages, the lack of widespread changes to cytokine genes in response to FcγR cross-linking was surprising. However, several factors must be taken into consideration. Firstly, ILC3 FcγR expression is confined to FcγRIII at rest and it expression level is considerably smaller compared to intestinal macrophages, which express the whole cohort of FcγRs in mice [69]. Therefore, IC-induced transcriptional changes are likely to be smaller, of a more directed nature, and may require longer stimulation times to attain significant levels. Secondly, these cells were obtained from the SI of uninflamed WT mice. It is possible that under inflammatory conditions, ILC3s are reprogrammed to respond to FcγR signalling in a different manner. In intestinal macrophages, inflammation had a profound effect on the transcriptional profile of these cells, but did not significantly alter the function of FcγR signalling. Therefore, it is likely that these transcriptional changes are reflective of both uninflamed and inflamed ILC3s. However, the magnitude of these changes may be significantly altered. Most intriguingly, a recent publication identified ILCs as existing in a primed state within tissues, with high basal expression of inflammatory cytokines. As such, changes in the processing of transcripts and proteins may be more relevant in these cells than changes at the transcriptional level of immune-associated genes themselves [557].

One cytokine that was significantly differentially expressed in response to IC was GM-CSF. GM-CSF is involved in myelopoiesis and granulocyte and MNP activation within tissues, and contributes to the inflammatory milieu in patients with IBD [413]. While the role of GM-CSF in chronic colitis is unclear and likely pleiotropic, several studies have demonstrated that this cytokine can promote detrimental inflammation through the activation of GI-resident granulocytes [297], [414]. Therefore, this raises the possibility that IC suppresses local ILC3-mediated inflammation by downregulation of GM-CSF expression. Given the induction of macrophage inflammatory programmes by IgG that promote granulocyte recruitment and activation, such as IL-1β production, this may act as a negative feedback loop following the emergence of local IgG. Whether this has a dominant effect *in vivo*, however, is unclear, given that *Fcgr2b*-deficient mice have elevated colonic GM-CSF levels compared to WT mice.

Of significant interest from these RNAseq studies is the induction of genes involved in ILC3 development, maintenance and activation. IC stimulation induced expression of several key genes involved in ILC development, including PLZF (encoded by *Zbtb16*), *Id2*, and *Rora*. Furthermore, genes encoding ILC3 cytokine and chemokine receptors, including *Il7r*, *Il23r*, and *Ccr9*, and transcriptional regulators promoting type 17 responses, such as *Ikzf3*, *Tbx21*, and *Ahr*, were induced by IC stimulation. AHR is particularly important in ILC3 biology: AHR deficiency significantly impairs ILC3 maintenance and activity within the GI tract, resulting in SFB expansion and Th17 activation [594]. Not only was AHR expression increased by IC, but also expression of its co-factor, ARNT.

Furthermore, several of these genes have been shown to cooperate, either in ILC biology or in the regulation of naïve $CD4^+$ T cell differentiation, for the induction of type 17 responses. For example, IL-23R signalling induces STAT3 phosphorylation, which subsequently cooperates with AHR for the induction of Th17-associated genes, including *Rorc* [606], [610]. Furthermore, STAT3 and AHR induce Aiolos, a Th17-inducing epigenetic modifier that silences Th1-associated genetic programmes, such as *Il2* expression [610]. In addition to the observed suppression of IL-2 and IL-2Rα expression, which drive Th1 polarisation, these results reinforce the notion that FcγR signalling acts to directly promote a ILC3 phenotype. Therefore, it is possible that elevated IL-22 expression *in vitro* following IC stimulation, and *in vivo* in *Fcgr2b*-deficient mice, is as a consequence of upregulation of AHR, STAT3 signalling, and Aiolos expression. As such, the dynamics of ILC3-to-ILC1 transition *in vivo* in these mice is of great interest. Currently in progress is the crossing of *Rorc*-Cre and *Fcgr2b*-floxed mouse strains to attempt to address some of these questions in the future.

An area of continued research is to investigate the effect of FcγR signalling on ILC1s, although this is hampered by the lack of distinct cell surface markers or access to reporter mice. If our hypothesis regarding ILC plasticity is correct, one might expect ILC1s to undergo transcriptional reprogramming towards an ILC3 phenotype in response to IC. However, whether so-called ex-ILC3 ILC1-like cells and bone fide ILC1s represent the same cell type is not clear. For example, given the role of FcγR signalling on the closely-related NK cell, one might expect IFNγ secretion by ILC1s in response to local IgG. In a similar vein, it should be noted that in this study, ILC3s were identified as Lin^- $CD45^{int}$ $ROR\gamma t^+$ cells using RORγt-GFP reporter mice. As such, RNAseq data represents a mix of all three ILC3 subsets. Whether FcγR cross-linking has different effects on ILC3 subsets, therefore, is not clear. LTi cells and $NKp46^+$ ILC3s are developmentally distinct, with the latter and ILC1s both deriving from the ILCP [550], [567]. Therefore, ILC3-to-ILC1 plasticity may be an attribute specific to the $NKp46^+$ subset, a distinction that cannot be resolved here.

5.9.6. Summary

In summary, in this chapter we have shown that ILC1s and ILC3s express FcγRIII and FcγRIIB under inflammatory conditions, that ICs indirectly promote IL-22 production and induce MHC-II expression by ILC3s, and ILC3-intrinsic FcγR signalling induces a transcriptional programme that reinforces ILC3 maintenance and functionality in detriment of ILC1 responses (Fig. 5.21). These results represent a new paradigm for sensing by ILCs, with direct regulation by the adaptive immune response, contrasting to the largely innate cell-derived cytokine mechanisms of stimulation described in the literature. This has potential consequences for IgG-mediated inflammation within the GI tract. However, whether these effects are unique to specific ILC3 subsets and are mirrored by transcriptional changes in ILC1s is not clear and the subject of future research.

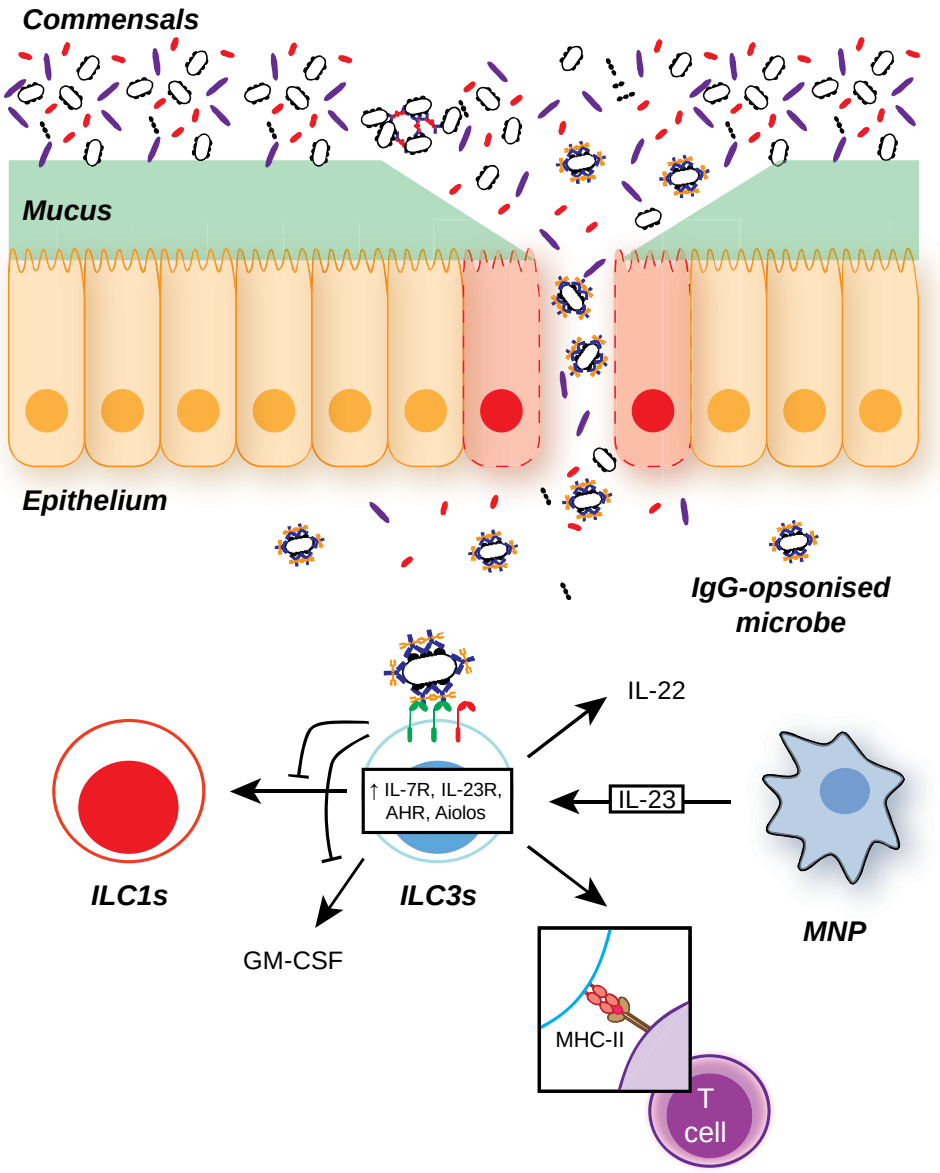

Figure 5.22. Chapter 5 graphical summary. ILC3s and ILC1s express activating FcγRIII and, under inflammatory conditions, upregulate expression of inhibitory FcγRIIB. FcγR signalling induces a transcriptional programme associated with ILC3 maintenance, IL-22 production, and MHC-II expression. Green receptor = activating FcγR; red receptor = FcγRIIB.

6. ILC-macrophage crosstalk mediates intestinal immune defence

6.1. Introduction and hypotheses

ILCs are heavily dependent on macrophage and DC-derived cytokines. Intestinal CX3CR1^{+} MNPs have been shown to drive ILC3 activation through the production of IL-23, IL-1β, and TL1A, with implications for defence against enteropathogens and intestinal pathology in T cell-transfer and anti-CD40 models of colitis [291], [292], [294], [359]. In contrast, the reciprocal contribution of different ILC3-derived cytokines to intestinal immunity is just beginning to be unravelled. In the context of intestinal infection, such as in *C. rodentium*, studies have focused on the role of ILC3-derived IL-22 in the regulation of the epithelial barrier and preventing the systemic dissemination of bacteria [545]. However, recent data has suggested that ILC3-derived GM-CSF is required for the survival of tolerogenic DCs within the colon [623], while also promoting the accumulation of pathogenic IL-23-producing granulocytes and monocytes within the colon in intestinal inflammation [297], [414], [415]. Furthermore, ILC3-derived IL-17A was shown to improve the anti-microbial capacity of macrophages by the induction of ROS and TNFα production [585]. Overall, however, little is known about how ILCs may coordinate other local and distal immune cells for the eradication of infection.

Following some experiments performed as part of the work for chapter 5, we hypothesised that ILC3-derived GM-CSF orchestrates intestinal macrophage recruitment and antibacterial function, playing a critical pro-survival role during infection. To address this, we firstly aimed to determine whether ILCs were a major source of intestinal GM-CSF and whether they were required for appropriate defence against *C. rodentium* infection and inflammation in DSS-induced colitis. Next, we investigated whether ILCs and GM-CSF were required for the appropriate recruitment and function of intestinal macrophages *in situ* and the mechanism by which this might occur. Finally, we sought to determine whether ILC3-derived GM-CSF initiated cross-talk via the induction of type 17-inducing cytokines by macrophages that boost GM-CSF production. Studies have demonstrated that GM-CSF *in vitro* can promote IL-1β production by BMDMs, through a combination of enhanced glycolysis and expression of a GTPase required for caspase-1-dependent pro-IL-1β processing [410], [411]. Whether intestinal macrophage cytokine production is similarly dependent on GM-CSF is poorly understood, while no studies exist on the role of ILC3-derived GM-CSF in this regard.

6.2. ILC depletion in *Citrobacter rodentium* infection

To determine the role of ILCs in protection against intestinal infection, *Citrobacter rodentium* infection, a murine model of human attaching-effacing *E coli* infection, was carried out in *Rag*2 KO mice treated with anti-CD90.2 IgG. We confirmed local ILC depletion by flow cytometry (Fig. 6.1): total colonic CD90.2$^+$ cells were reduced in the following anti-CD90.2 IgG treatment. In order to overcome confounding effects of anti-CD90.2 IgG treatment on the staining of CD90.2 by flow cytometry, ILCs were also quantified based on production of IL-22 and IL-17A, both showing a similar reduction in ILCs following treatment (Fig. 6.1). Mice treated with anti-CD90.2 IgG exhibited greater bacterial burden at day 7 post-infection, with increased CFUs locally within the caecum compared to mice treated with control IgG (Fig. 6.2A). Furthermore, anti-CD90.2 IgG treatment increased hepatic dissemination of bacteria, while few bacteria were detected in the spleen (Fig. 6.2B). Therefore, ILC depletion was successful, with these cells being essential for the maintenance of appropriate anti-bacterial immunity within the GI tract.

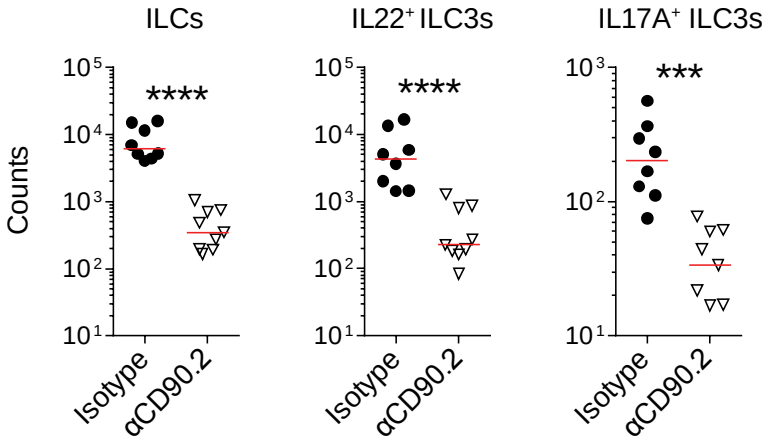

Figure 6.1. Anti-CD90.2 IgG treatment successfully depletes intestinal ILCs. Flow cytometric quantification of colonic CD90.2$^+$ SCA-1$^+$ ILCs, and IL-22/IL-17A-producing ILCs at day 7 post-infection. Data are representative of three independent experiments. *P* values were calculated using the nonparametric Mann-Whitney test. **** $P < 0.0001$.

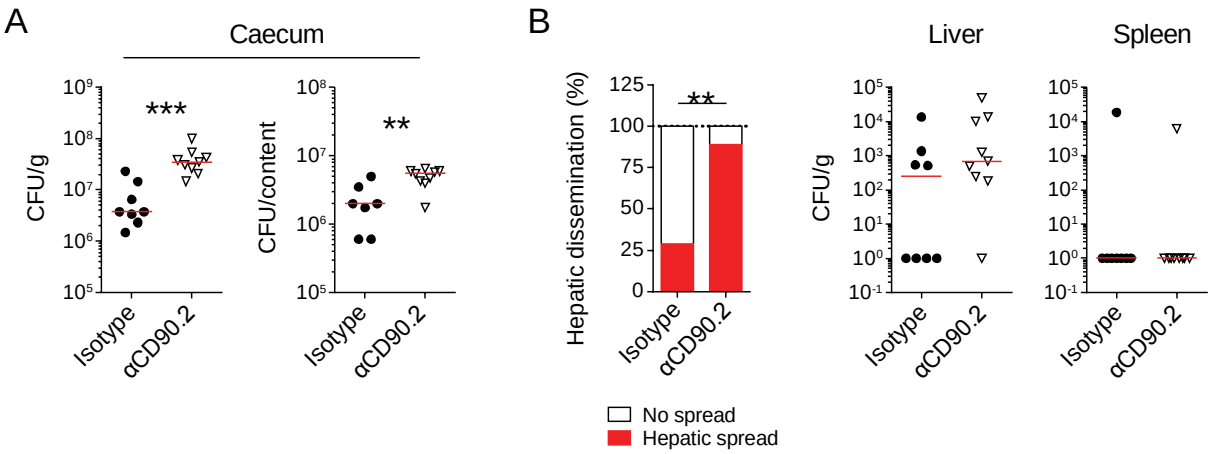

Figure 6.2. ILC depletion enhances bacterial burden in *Citrobacter rodentium* infection. (A) Caecal CFU analysis at day 7 post *C. rodentium* infection in mice treated with anti-CD90.2 IgG or control IgG. (B) Analysis of bacterial systemic dissemination at day 7 post *Citrobacter rodentium* infection. Hepatic dissemination quantification is pooled from two datasets. Otherwise, data are representative of two independent experiments. *P* values were calculated using the nonparametric Mann-Whitney test (A) or a Chi-squared test (B). ** $P < 0.01$; *** $P < 0.001$.

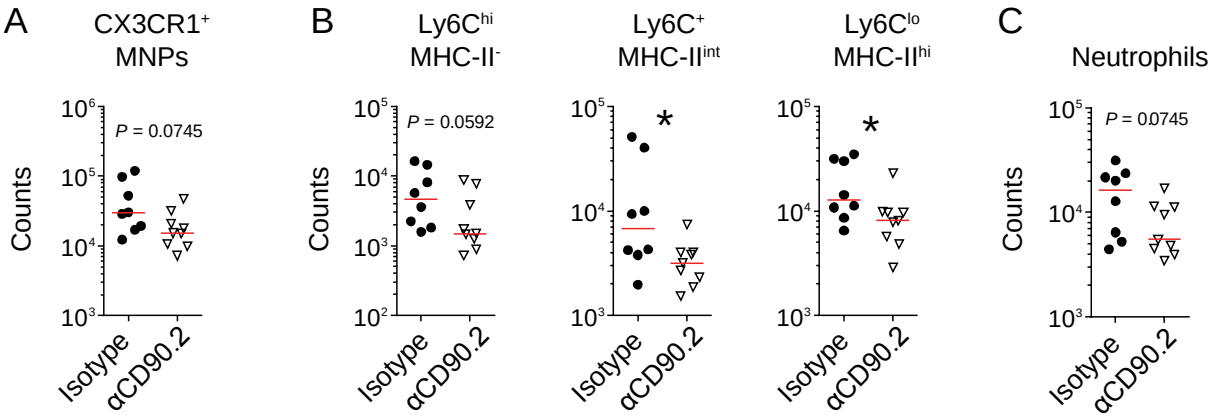

Figure 6.3. ILC depletion reduces intestinal CX3CR1⁺ MNPs. Flow cytometric quantification of total colonic CX3CR1⁺ MNPs (A), MNP subsets (B), and neutrophils (C) at day 7 post infection in mice treated with anti-CD90.2 IgG or control IgG. Data are representative of two independent experiments. *P* values were calculated using the nonparametric Mann-Whitney test. * $P < 0.05$.

Increased bacterial dissemination could result from impaired barrier function, likely due to the loss of IL-22-producing ILC3s, but may also be due to defective bacterial clearance by immune cells, including macrophages and neutrophils. We, therefore, sought to profile the phenotype of other intestinal immune cells in the absence of ILCs. Analysis of CX3CR1⁺ MNPs by flow cytometry demonstrated a trend towards a reduction in total MNPs in the absence of ILCs (Fig.

6.3A). Subset analysis using Ly6C and MHC-II as waterfall markers demonstrated a reduction in the total number of MHC-II^{+} monocytes and Ly6C^{lo} MHC-II^{hi} macrophages following anti-CD90.2 treatment (Fig. 6.3B). We also observed a trend towards a reduction in total colonic neutrophil numbers in the absence of ILCs (Fig. 6.3C). Furthermore, analysis of pro-IL-1β production by CX3CR1^{+} MNP subsets demonstrated a striking defect in cytokine production in the absence of ILCs (Fig. 6.4A). This was most apparent in the macrophage population, but also detected to a lesser extent in the MHC-II^{-} and MHC-II^{+} monocyte populations (Fig. 6.4B). Given the increased local and systemic bacterial burden, it may seem surprising that macrophages appear unresponsive.

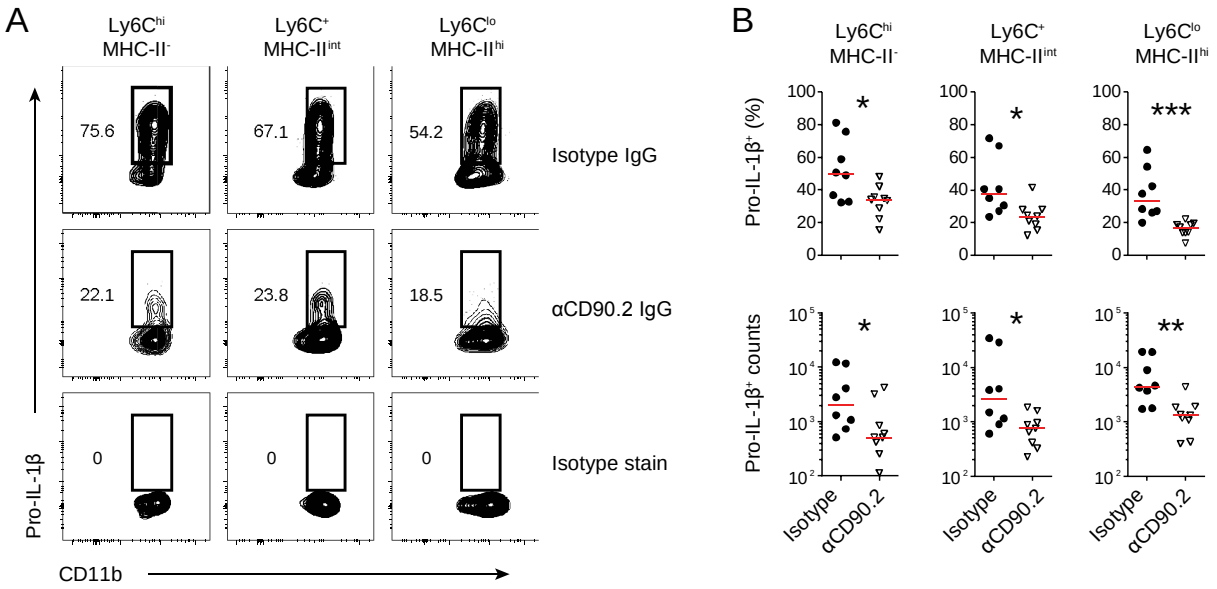

Figure 6.4. Defective MNP IL-1β production in the absence of ILCs. (A) Intracellular flow cytometric staining of pro-IL-1β-expressing colonic MNP subsets at day 7 post *C. rodentium* infection in mice treated with anti-CD90.2 IgG or control IgG. (B) Quantification of frequency and absolute number of pro-IL-1β^{+} MNP subsets. Data are representative of two independent experiments. *P* values were calculated using the nonparametric Mann-Whitney test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Next, we sought to determine whether other IL-1β-producing cells were similarly affected. While intracellular cytokine staining demonstrated that CX3CR1^{+} MNPs are the major source of intestinal IL-1β production in response to *C. rodentium* infection, cytokine production was also identified in CX3CR1^{-} Ly6C/G^{hi} neutrophils and CX3CR1^{-} Ly6C/G^{-} CD11b^{+} DC subsets (Fig. 6.5A). Cytokine production by CX3CR1^{+} MNPs was greatest in terms of number but also on a per-cell basis, as determined by pro-IL-1β GMFI (Fig. 6.5B). The total number of pro-IL-1β-producing cells was reduced in the absence of ILCs (Fig. 6.5C), with subset analysis demonstrating that, while neutrophils were largely unaffected by ILC depletion, pro-IL-1β

production by CX3CR1^{+} Ly6C^{lo} MHC-II^{hi} was the most significantly affected by anti-CD90.2 IgG treatment (Fig. 6.5D). Reduced cytokine production was also observed in the DC population, suggesting a predominant defect in cytokine production by tissue-resident macrophages and DCs in the absence of ILCs.

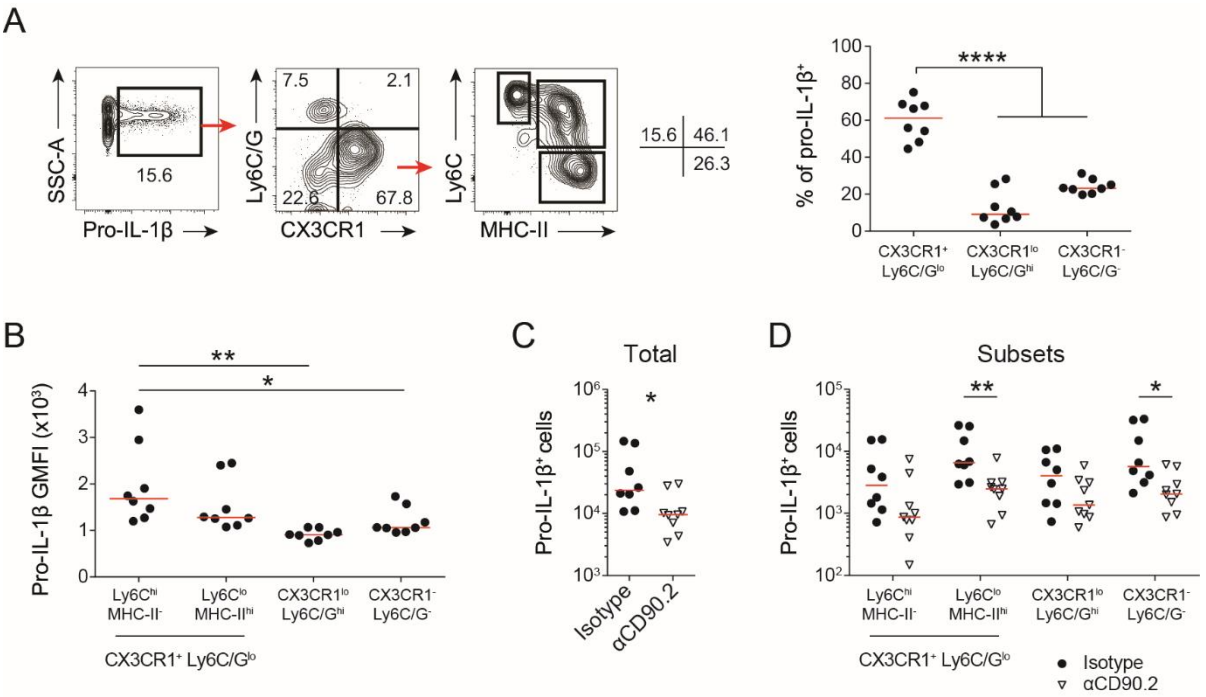

Figure 6.5. Analysis of ILC depletion on all IL-1β-producing immune cells. (A) Unbiased analysis of pro-IL-1β-producing immune cells by flow cytometry at day 7 post infection. (B) Quantification of pro-IL-1β GMFI across immune cell subsets identified in A. (C) Quantification of total colonic IL-1β-producing immune cells at day 7 post-infection in mice treated with anti-CD90.2 IgG or control IgG. (D) Quantification of IL-1β-producing subsets in A in mice treated with anti-CD90.2 IgG or control IgG at day 7 post-infection. Data are representative of two independent experiments. *P* values were calculated using a one-way ANOVA (A, B) or the nonparametric Mann-Whitney test. * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$.

Consistent with previous pro-IL-1β analysis, neutrophils were largely unaffected by the absence of ILCs: while there was a trend towards reduced numbers of neutrophils in the absence of ILCs, CD11b GMFI (a marker of neutrophil activation), and pro-IL-1β production by these cells was unaffected (Fig. 6.6).

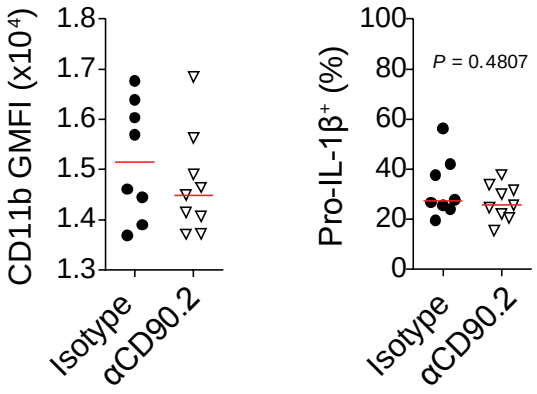

Figure 6.6. Neutrophils are unaffected by ILC depletion. Flow cytometric analysis of neutrophil phenotype at day 7 post-infection in mice treated with anti-CD90.2 IgG or control IgG. Data are representative of two independent experiments. *P* values were calculated using the nonparametric Mann-Whitney test.

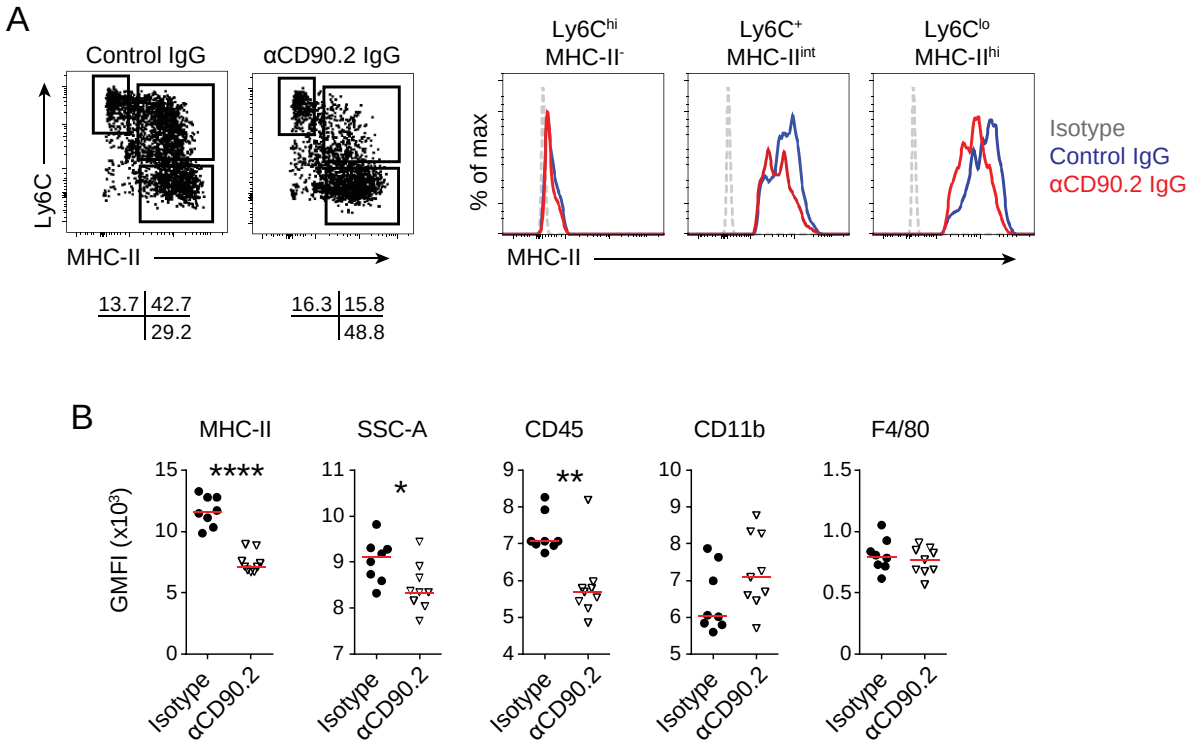

Figure 6.7. ILC depletion reduces MHC class II expression by intestinal macrophages. (A) Flow cytometry showing MHC-II expression by subsets of the colonic monocyte waterfall at day 7 post-infection in mice treated with anti-CD90.2 IgG or control IgG. (B) Analysis of cell surface markers on CX3CR1^{+} Ly6C^{lo} MHC-II^{hi} macrophages at day 7 post-infection. Data are representative of two independent experiments. *P* values were calculated using the nonparametric Mann-Whitney test. * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$.

As well as cytokine production, a variety of other macrophage markers were influenced by ILC depletion. Analysis of MHC-II expression in the CX3CR1^{+} MNP waterfall demonstrated an impaired upregulation of MHC-II expression by monocytes following anti-CD90.2 IgG treatment (Fig. 6.7A). Furthermore, macrophages exhibited reduced side scatter and CD45 expression (Fig. 6.7B), consistent with a more general cellular defect rather than solely influencing IL-1β production.

Therefore, ILCs play an essential role in the maintenance of intestinal immunity, particularly through the maintenance of an activated macrophage phenotype. Following anti-CD90.2 IgG administration, caecal and systemic bacterial burdens where increased, while cytokine production by intestinal CX3CR1^{+} MNPs was inhibited. Furthermore, reductions in total MNP numbers were also observed, with defects most pronounced within the Ly6C^{lo} MHC-II^{hi} mature macrophage population.

6.3. ILC depletion in DSS-induced colitis

To address whether this defective macrophage phenotype in the absence of ILC3s was specific to *Citrobacter rodentium* infection, or whether it was applicable to other forms of intestinal inflammation, *Rag2* KO mice were treated with anti-CD90.2 or control IgG and subjected to an acute 7-day course of DSS-induced colitis.

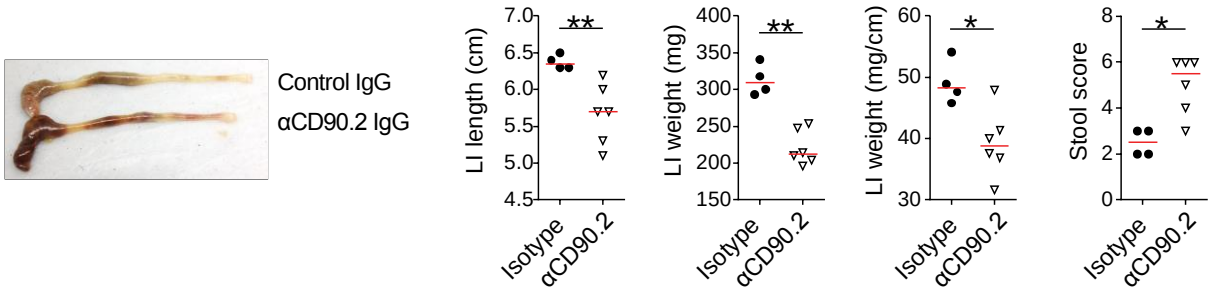

Figure 6.8. Anti-CD90.2 treatment augments disease severity in DSS-induced colitis. Clinical parameters after an acute 7-day course of DSS administration in mice treated with anti-CD90.2 or control IgG. *P* values were calculated using the nonparametric Mann-Whitney test. * $P < 0.05$; ** $P < 0.01$.

Following anti-CD90.2 IgG treatment, *Rag2* KO mice exhibited worsening clinical features of inflammation, including reduced colon length and enhanced stool score, with pronounced gastrointestinal bleeding (Fig. 6.8). Curiously, however, colon weight was significantly reduced in the absence of ILCs, a clinical feature generally used to demonstrate *reduced* disease severity. Therefore, while gastrointestinal bleeding was more severe, local tissue inflammation may be reduced in the absence of ILCs, reflecting perhaps the differing roles played by ILC-derived mediators in an inflammatory response.

Once again, ILC depletion was confirmed by flow cytometry (Fig. 6.9). We sought to profile GM-CSF-producing cells by flow cytometry. CD90.2$^+$ SCA-1$^+$ ILCs were identified as the major source of colonic IL-22 and GM-CSF (Fig. 6.9A). Furthermore, anti-CD90.2 IgG treatment resulted in the reduction of total IL-22 and GM-CSF-producing cells (Fig. 6.9B), as well as IL-22 and GM-CSF-producing ILC3s, the major GI-resident ILC subset producing these cytokines *in vivo*.

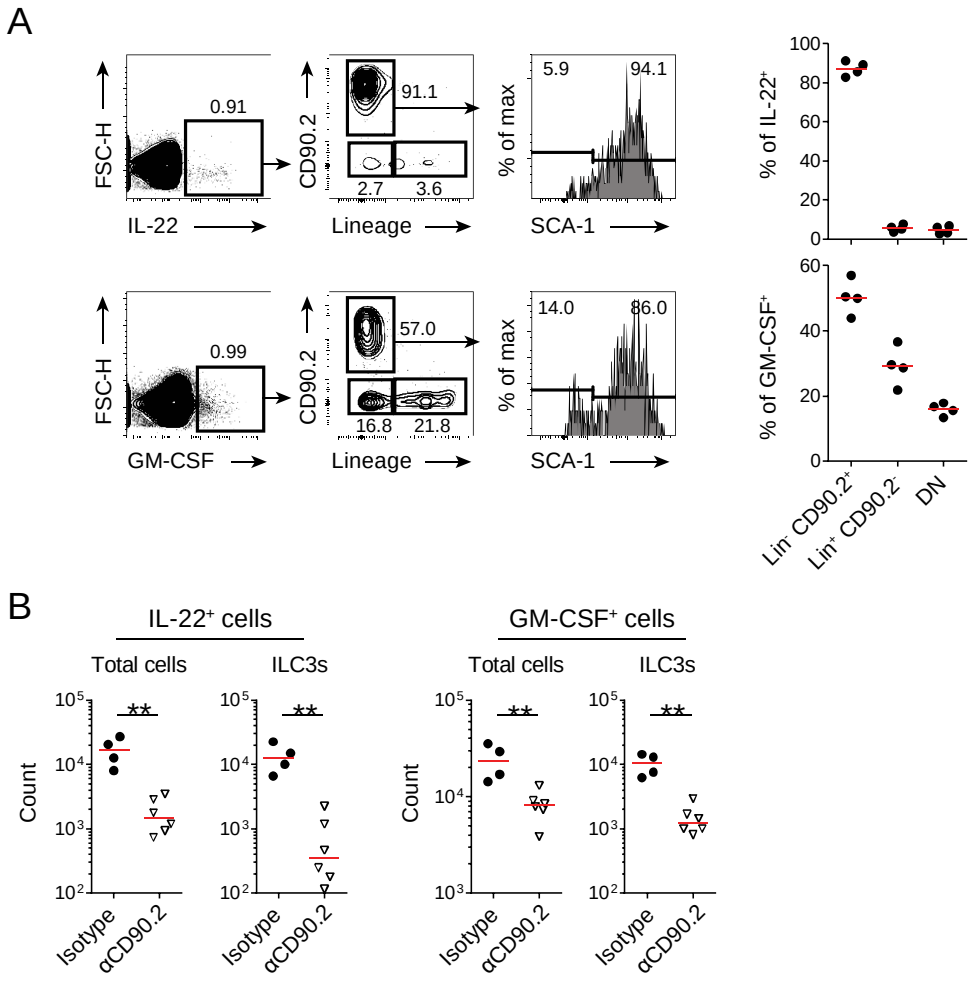

Figure 6.9. ILCs are the major source of IL-22 and GM-CSF and are depleted following anti-CD90.2 IgG treatment. (A) Intracellular cytokine staining for IL-22 and GM-CSF-producing cells by flow cytometry at day 7 post-DSS. (B) Depletion of total and CD90.2$^+$ SCA-1$^+$ IL-22- and GM-CSF-producing cells following anti-CD90.2 IgG or control IgG administration at day 7 post-DSS. Data are representative of three independent experiments. *P* values were calculated using the nonparametric Mann-Whitney test. ** $P < 0.01$.

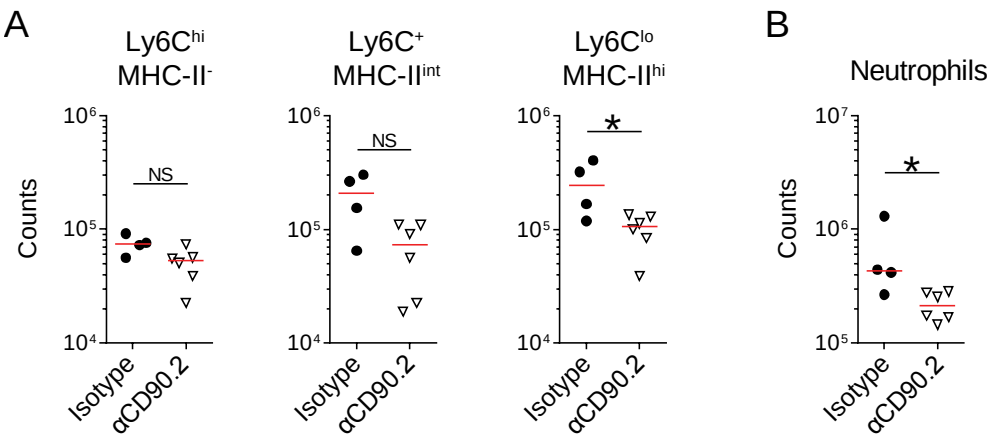

Figure 6.10. Reduced macrophage and neutrophil numbers in the absence of ILCs. (A, B) Quantification of CX3CR1$^+$ MNP subsets (A) and neutrophil (B) by flow cytometry in colons of mice after a 7-day course of DSS. Data are representative of three independent experiments. *P* values were calculated using the nonparametric Mann-Whitney test. * $P < 0.05$.

Consistent with reduced immune infiltrate into the colonic LP, profiling of macrophage numbers by flow cytometry once again demonstrated reduction in these cells in the absence of ILCs (Fig. 6.10A). ILC depletion once again primarily affected the macrophage subset of CX3CR1$^+$ MNPs, with a less pronounced effect on monocytes. While trending in *Citrobacter rodentium* infection, neutrophil numbers were significantly reduced in the colonic LP after DSS administration in the absence of ILCs (Fig. 6.10B).

Pro-IL-1β production by CX3CR1$^+$ MNPs was higher in DSS-induced colitis compared to *C. rodentium* infection, likely reflecting the broader inflammatory response induced by epithelial cell toxicity. However, cytokine production was once again significantly reduced in the absence of ILCs, with an enhanced effect as Ly6C^{hi} monocytes matured into Ly6C^{lo} MHC-II^{hi} macrophages (Fig. 6.11A). In addition to trends in the reduction of all CX3CR1$^+$ MNP subsets (Fig. 6.10A), this resulted in significantly reduced numbers of all cytokine-producing CX3CR1$^+$ MNP subsets (Fig. 6.11B).

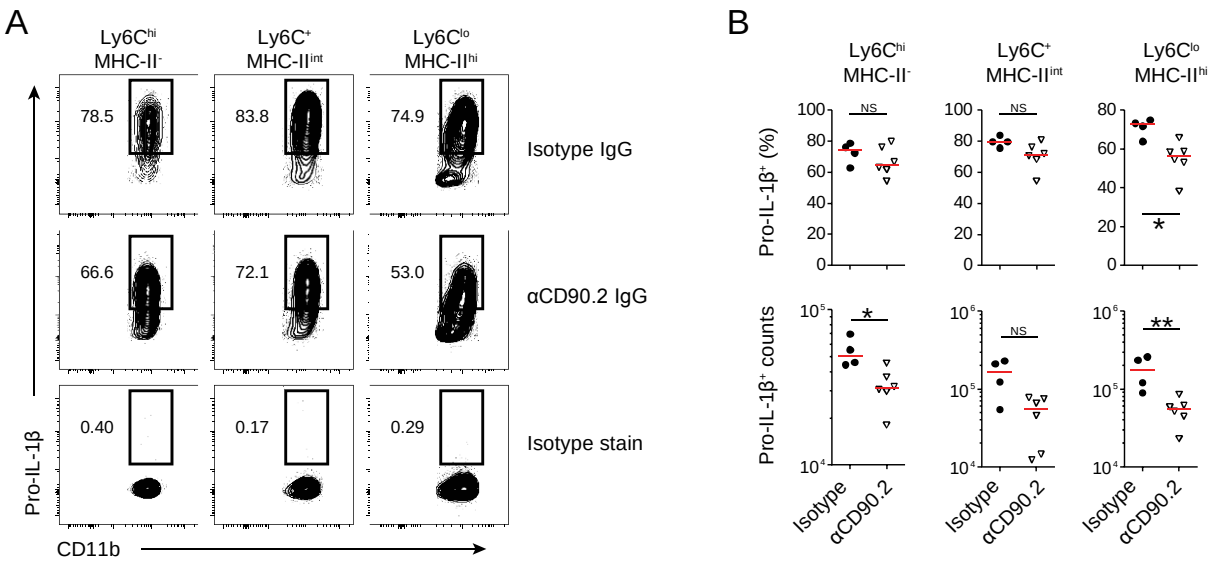

Figure 6.11. Defective macrophage IL-1β production in the absence of ILCs. (A, B) Intracellular flow cytometry staining of pro-IL-1β-producing MNP subsets, showing representative plots (A) and quantification (B) at day 7 post DSS administration. Data are representative of three independent experiments. *P* values were calculated using the nonparametric Mann-Whitney test. * $P < 0.05$; ** $P < 0.01$.

In summary, ILC depletion results in enhanced disease severity following acute DSS-induced colitis, with reduced colon length and worsening stool scores. However, the reduction in intestinal ILCs also dampens the local inflammatory immune cell response, as highlighted by reduced intestinal macrophage and neutrophil numbers, dysfunctional IL-1β production, and a globally reduced inflammatory response following anti-CD90.2 IgG treatment versus mice receiving control IgG. Therefore, enhanced disease severity is likely a combination of enhanced commensal infiltration and dissemination from the intestinal lumen into host tissues, as well as potentially differing roles of ILC-derived products in the coordination of tissue immunity and homeostasis, such as IL-22 in barrier function and GM-CSF in myeloid cell maturation. These results support the observations made following CD90.2 depletion in *C. rodentium* infection, and suggest common immune-mediated inflammatory mechanisms regulated by ILCs.

6.4. Potential mechanisms for macrophage regulation by ILCs

We hypothesised that the observed defects in macrophage number and activation state in the absence of ILCs could have a number of explanations (Fig. 6.12). (1) Defects in the recruitment of monocytes to the GI tract are consistent with the reduction in Ly6C^{hi} cells observed within the LP in both models. (2) Reduced macrophage MHC-II and CD45 expression, side scatter, and cytokine production could result from impaired monocyte

maturation. (3) ILC-derived mediators could directly prime macrophages for cytokine production. (4) ILCs may be required for the survival of macrophages within the tissue. Alternatively, the observed macrophage phenotype could be due to a combination of these effects.

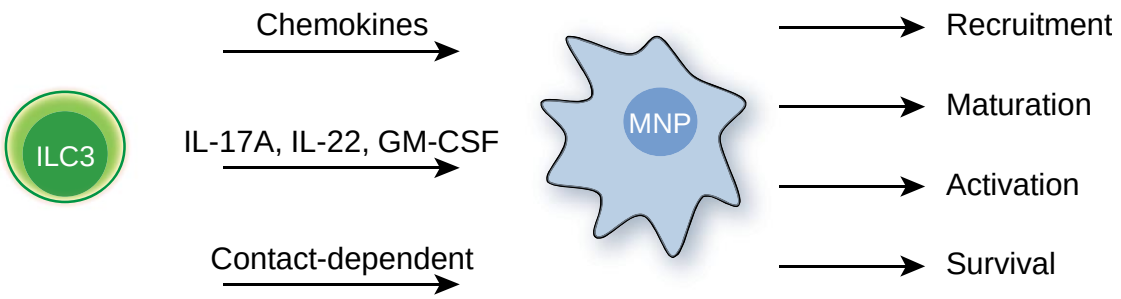

Figure 6.12. Potential macrophage-immunomodulatory functions of ILC3s. ILC3s may regulate macrophage recruitment and activation within the colonic lamina propria through a variety of contact-dependent and contact-independent mechanisms.

6.5. Chemokine expression by intestinal ILCs

Little is known about how ILCs may contribute to immune cell recruitment via the secretion of chemokines. To address this question, we investigated chemokine gene expression in transcriptomics datasets of small intestinal ILC subsets from the Immgen consortium.

Several chemokine transcripts were identified in SI ILC subsets. Of note, *Ccl3*, *Cxcl10*, and *Cx3cl1* were expressed by SI ILC3s (Fig. 6.13). CX3CL1 is the ligand for CX3CR1 – the major MNP-associated chemokine receptor in the gut. CX3CR1 is expressed by tissue-resident macrophages throughout the body, including microglia, and intestinal and pulmonary macrophages: tissues in which ILCs are known to reside. This, therefore, raises the possibility that ILCs are required for maintenance of tissue-resident macrophages throughout the body. CXCL10 and CCL3 are also known to drive monocyte recruitment via binding to CXCR3 and CCR1/4/5, respectively. Shown in Figure 6.13 are chemokines expressed at moderate levels in at least one ILC subset. The classical monocyte-recruiting chemokine CCL2 is not included, as this is very lowly expressed by ILCs.

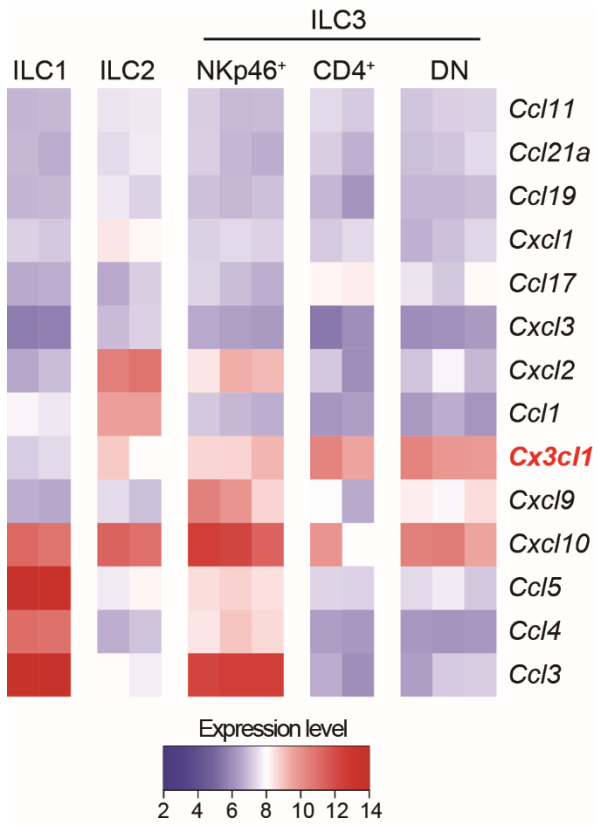

Figure 6.13. ILCs express macrophage-recruiting chemokines. Heatmap showing transcriptomics analysis of chemokine expression by small intestinal ILC subsets (GSE37448).

6.6. Cytokine analysis in ILC-depleted colons

ILC3s are particularly abundant within the GI tract and important mediators of local immunity through the production of IL-22, IL-17A, and GM-CSF. While an expanding body of literature has demonstrated the role of IL-22 in mediating intestinal barrier reinforcement in several models of intestinal inflammation and homeostasis [545], [573], [624], the study of how ILCs regulate proximal tissue-resident immune cells through the production of cytokines, such as GM-CSF and IL-17A, is still in its infancy. We hypothesised that ILC3-derived IL-17A and GM-CSF may play a role in the local activation of macrophages. Therefore, we decided to profile changes in cytokine gene expression in whole colon tissue by qPCR following CD90.2 depletion in *C. rodentium* infection and DSS-induced colitis.

A significant reduction in global *Csf2* levels was seen following CD90.2 depletion in *C. rodentium* infection (Fig. 6.14). IL-17A expression was very lowly expressed within the colon and not detected by qPCR. Curiously, *Il6* expression was increased in the absence of ILCs. In DSS-induced colitis, the expression of several inflammatory cytokines was significantly reduced following anti-CD90.2 IgG treatment, including *Il1b*, *Tnf*, *Il22*, and a pronounced reduction in *Csf2* (Fig. 6.15). Therefore, CD90.2 depletion is consistently associated with a

reduction in intestinal GM-CSF expression levels, with GM-CSF being significantly more highly expressed that IL-17A.

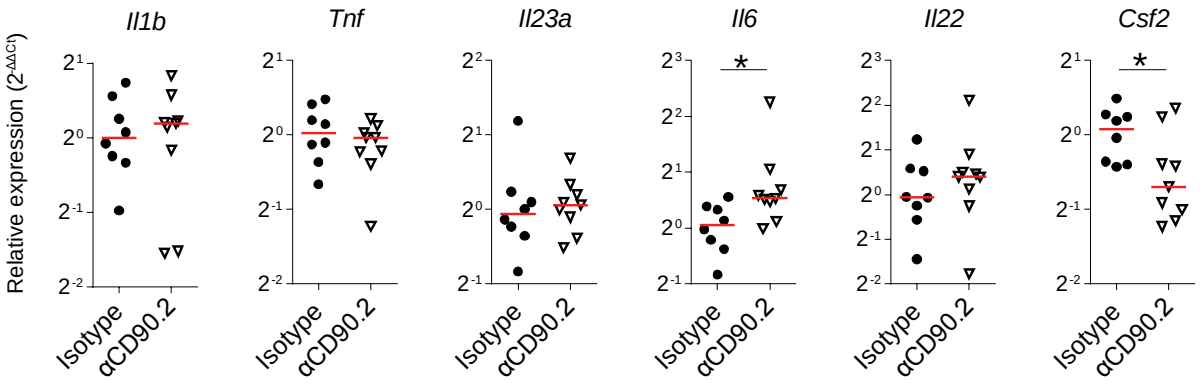

Figure 6.14. ILC depletion reduces intestinal GM-CSF levels in *C. rodentium* infection. qPCR analysis of cytokine production in whole colon tissue at day 7 post-infection. *Il17a* levels were too low and were not detected. Data represent a single experiment. *P* values were calculated using the nonparametric Mann-Whitney test. * $P < 0.05$.

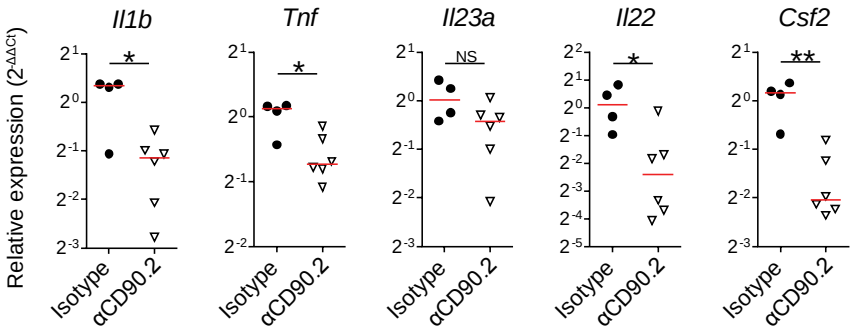

Figure 6.15. Reduced inflammatory cytokine profile in the absence of ILCs during DSS-induced colitis. qPCR of whole colonic tissue from mice receiving anti-CD90.2 IgG or control IgG after a 7-day course of DSS. *Il17a* levels were too low and not detected. Data are representative of three independent experiments. *P* values were calculated using the nonparametric Mann-Whitney test. * $P < 0.05$; ** $P < 0.01$.

6.7. *In vitro* macrophage GM-CSF stimulation

We hypothesised, given the phenotype of macrophages in the absence of ILCs, the pronounced depletion of colonic GM-CSF levels in the gut following ILC depletion, and the role of GM-CSF in regulating macrophage inflammatory responses, that intestinal ILC3s could coordinate local macrophage immune responses by production of GM-CSF. This priming could enhance cytokine production, induce anti-microbial responses, such as ROS and NO production, and alter the metabolic profile of macrophages leading to enhanced anti-commensal and anti-pathogen immunity.

Of ILC3-derived cytokine receptors, intestinal macrophages express highest levels of GM-CSF receptors (Fig. 6.16A). These are present in all CX3CR1^{+} MNP subsets, regardless of inflammation. Macrophages also expressed significant levels of IL-17RA, although expression levels of other IL-17R-forming subunits were much lower. IL-22R levels were very low, consistent with their expression predominantly on non-haematopoietic cells. In keeping with an important role for GM-CSF, we found that priming of BMDMs with 20 ng/ml GM-CSF for 24 h resulted in a significant increase in inflammatory cytokine production in response to LPS stimulation compared to unprimed BMDMs (Fig. 6.16B). This effect was most pronounced for IL-1β and IL-23, with smaller yet significant effects also observed on CXCL chemokine production. IL-1β production is classically thought to require two signals, a priming signal, such as LPS, that induces *Il1b* expression, and a second signal that results in inflammasome assembly. These results demonstrate that LPS and GM-CSF in combination are sufficient for IL-1β expression, processing and secretion by macrophages. Furthermore, the pronounced increase in IL-23 expression, a cytokine not thought to require such two-step signalling, suggests other priming mechanisms that may be contributing to macrophage responsiveness.

GM-CSF priming also resulted in enhanced production of CXCL1 and CXCL2 by BMDMs stimulated with O-IC (Fig. 6.16C), but no IL-1β protein production was observed under these conditions. Therefore, GM-CSF enhance cytokine production by macrophages in response to TLR and FcγR stimulation, with the exact inflammatory profile depending on the secondary stimulus.

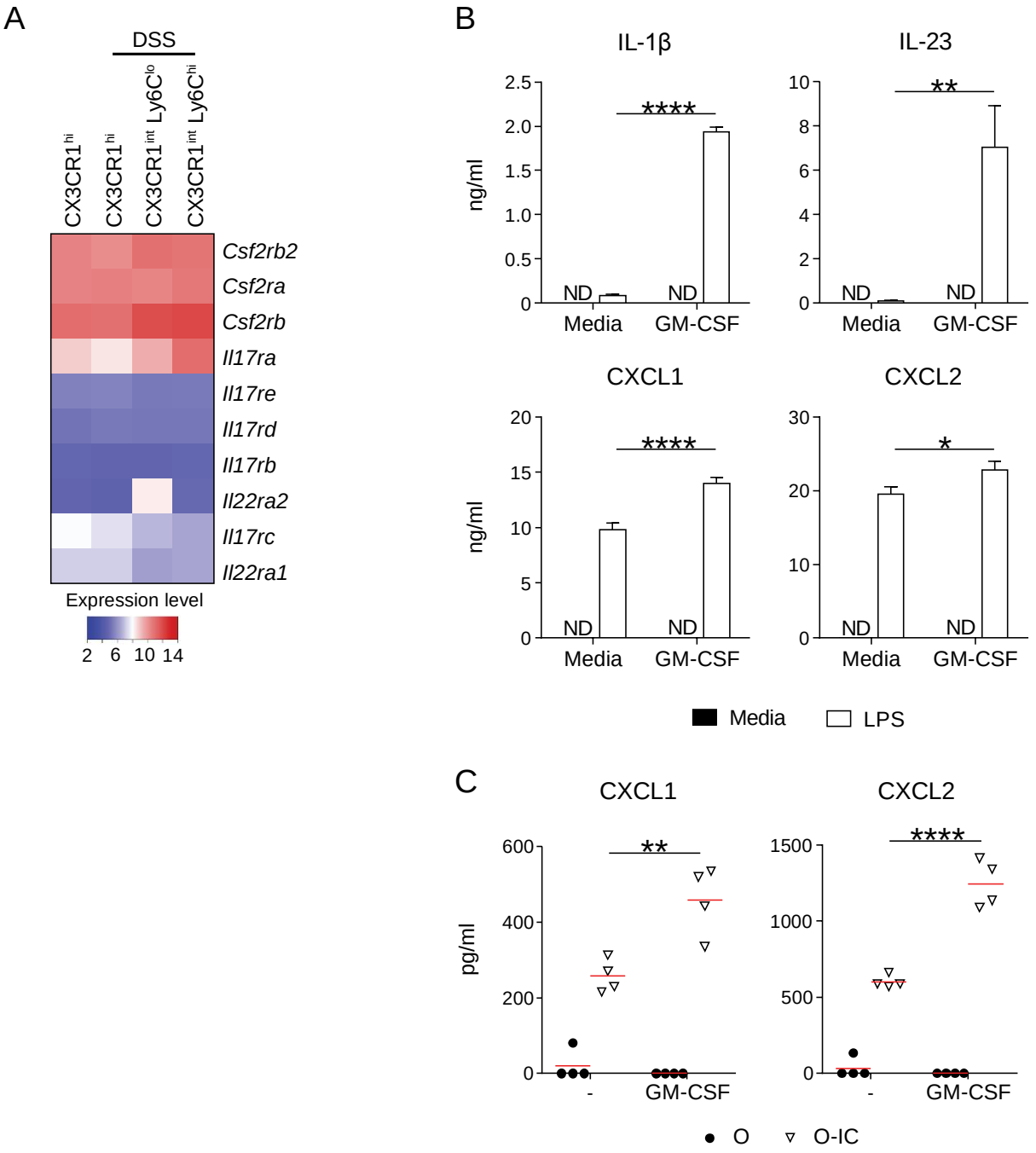

Figure 6.16. GM-CSF promotes inflammatory cytokine production by macrophages. (A) Heatmap showing transcriptomics analysis of ILC-derived cytokine receptors expressed by intestinal macrophage subsets (GSE42101). (B) ELISA of cytokine and chemokine production by BMDMs primed for 24 h with 20 ng/ml GM-CSF prior to 16 h stimulation with LPS. (C) Chemokine production by GM-CSF-primed BMDMs stimulated for 16 h with O or O-IC. *n* = 4 per group. Data are representative of three independent experiments. *P* values were calculated using the parametric Student's *t* test. * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$.

6.8. GM-CSF neutralisation in DSS-induced colitis

Given the prominent effect of GM-CSF on macrophage cytokine production *in vitro*, we sought to determine whether GM-CSF neutralisation *in vivo* would have a similar effect to ILC anti-CD90.2 depletion in the suppression of macrophage cytokine production and the clinical severity of DSS-induced colitis.

Rag2 KO mice were administered with anti-CD90.2 IgG, anti-GM-CSF IgG or control IgG at days 0 and 3 on a 7-day DSS protocol. At endpoint, ILC-depleted mice lost significantly less weight compared to mice receiving control IgG (Fig. 6.17A). Anti-GM-CSF IgG administration resulted in reduced weight loss compared to control animals, although this reduction was not as large as CD90.2-depleted mice. Furthermore, analysis of colon parameters demonstrated increased colon length and reduced colon weight in the absence of ILC3s (Fig. 6.17B). Curiously, increased colon length in the absence of ILCs is opposite to what was observed in Figure 6.9. While not significant, there was a trend towards increased colon length and reduced weight following anti-GM-CSF administration. Therefore, anti-GM-CSF IgG administration can partially recapitulate the improved disease progression observed by ILC depletion.

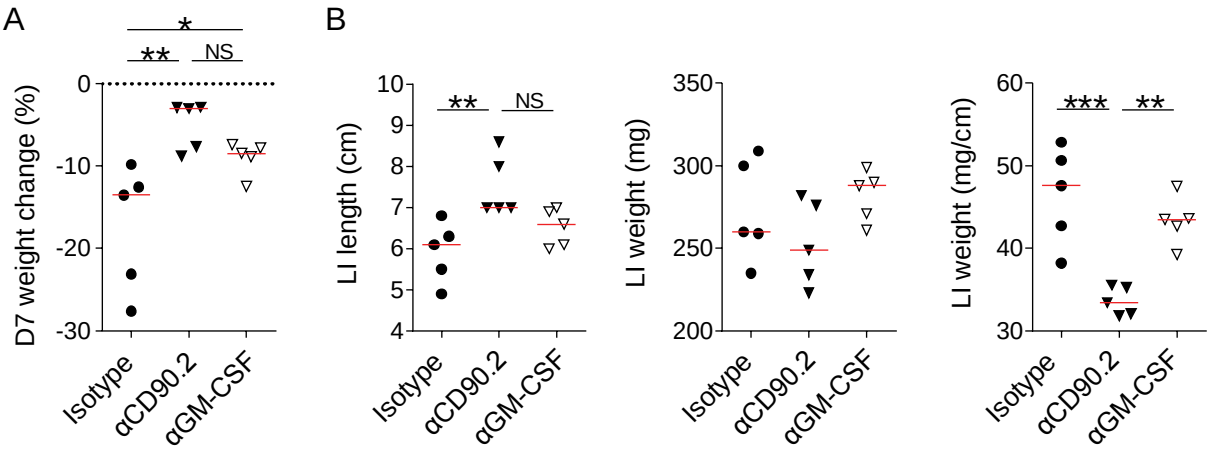

Figure 6.17. Anti-GM-CSF IgG administration partially dampens colitis severity. Final weight change (A) and colon clinical parameters (B) at day 7 post-DSS administration in *Rag2* KO mice receiving anti-CD90.2, anti-GM-CSF, or control IgG at days 0 and 3. Data are representative of two independent experiments. *P* values were calculated using the nonparametric Mann-Whitney test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Analysis of MNP and neutrophil numbers within the inflamed colonic LP by flow cytometry demonstrated a reduction in all MNP subsets with anti-CD90.2 IgG administration compared to controls, as previously observed (Fig. 6.18A). Anti-GM-CSF IgG administration did not significantly alter MNP numbers compared to controls. Therefore, ILC-mediated recruitment

of monocytes appears to be GM-CSF-independent. Consistent with previous data, ILC depletion once again resulted in a significant reduction of colonic neutrophils (Fig. 6.18B). In this case, anti-GM-CSF IgG administration trended towards reduced neutrophil infiltration, consistent with the known roles of GM-CSF on granulopoiesis [415] and our data demonstrating effects of GM-CSF on macrophage chemokine production. However, the variability of this group makes interpretation difficult.

Next, we sought to investigate how GM-CSF depletion may alter the cytokine profile of macrophages. Unlike monocyte recruitment, which is likely to occur secondarily to effects of GM-CSF on other cells, we have previously demonstrated that GM-CSF can directly influence LPS-induced inflammatory cytokine production by macrophages. Once again, we observed a significant reduction in pro-IL-1β production by intestinal CX3CR1^{+} MNPs in the absence of ILCs, with the most profound effect on mature MHC-II^{hi} macrophages (Fig. 6.19). Interestingly, anti-GM-CSF administration did not significantly reduce the percentage of pro-IL-1β-expressing MNP subsets, although this was trending towards a reduction in MHC-II^{hi} macrophages. In contrast, pro-IL-1β GMFI was significantly reduced in intestinal MHC-II^{hi} macrophages. Therefore, while the number of cytokine-producing cells did not change significantly, the amount of pro-IL-1β expressed per cell was significantly reduced.

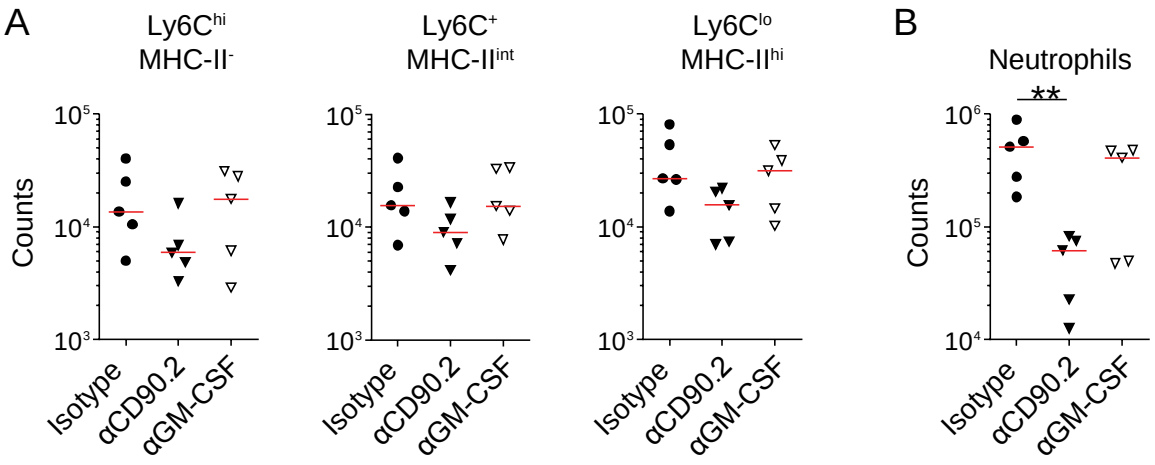

Figure 6.18. GM-CSF neutralisation has no significant effects on neutrophil and monocyte recruitment. Flow cytometric analysis of colonic CX3CR1^{+} MNP subsets (A) and neutrophils at day 7 post-DSS administration. Data are representative of three independent experiments. *P* values were calculated using the nonparametric Mann-Whitney test. ** $P < 0.01$.

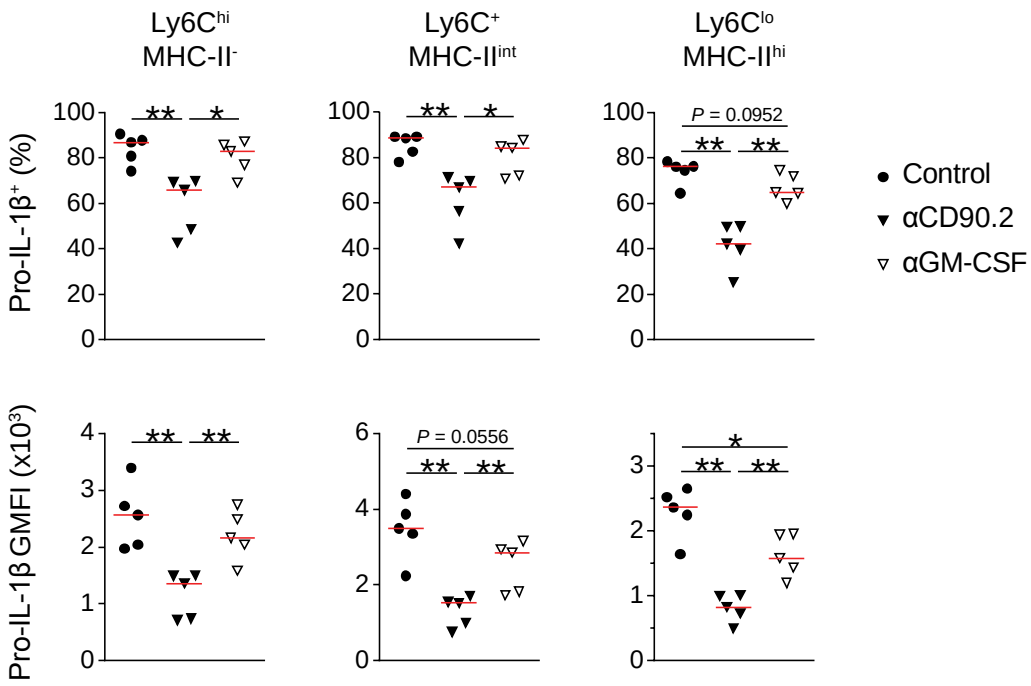

Figure 6.19. GM-CSF neutralisation reduces intestinal macrophage IL-1β production. Analysis of pro-IL-1β production by CX3CR1$^+$ MNP subsets at day 7 post-DSS administration by flow cytometry. Data are representative of three independent experiments. *P* values were calculated using the nonparametric Mann-Whitney test. * $P < 0.05$; ** $P < 0.01$.

In summary, GM-CSF neutralisation resulted in improved clinical disease progression and reduced macrophage IL-1β expression, although these effects were not as large as those induced by ILC depletion. Furthermore, GM-CSF neutralisation did not affect macrophage numbers, although there was a trend towards a reduction in neutrophils. Therefore, GM-CSF appears to contribute to several elements of the phenotype induced by pan-ILC depletion, including the activation of macrophages *in situ*. Whether additional elements contribute to macrophage activation in addition to GM-CSF or whether an alternative anti-GM-CSF IgG dosing strategy is required to phenocopy pan-ILC depletion is not clear at present, but is an active area of investigation.

6.9. Potential mechanisms for GM-CSF-mediated macrophage activation

GM-CSF may contribute to macrophage cytokine production through several mechanisms:

- Altered TLR and FcγR expression
- Enhanced pro-IL-1β processing
- Production of secondary mediators that induce macrophage activation
- Altered metabolic profile.

To answer this, we performed GSEA on a transcriptomics dataset of M-CSF versus GM-CSF-derived human MDMs (Fig. 6.20). GM-CSF resulted in enrichment of 49 out of 50 Hallmarks pathways (data not shown), with notable examples including *Glycolysis*, *Hypoxia*, *ROS pathway*, and *Inflammatory response* (Fig. 6.20A). This is consistent with an altered metabolic profile and enhanced anti-microbial immune response in GM-CSF-primed cells. Analysis of the core enrichment of *Glycolysis* genes demonstrated an enrichment in several key glycolysis enzymes, including lactate dehydrogenase (*LDHA*) and hexokinase 2 (*HK2*) (Fig. 6.20B). While not included in the *Glycolysis* Hallmarks gene list, glucose transporters are also known to be key regulators of glycolysis, including GLUT1 (*SLC2A1*) [411]. We found that GM-CSF-derived MDMs had enhanced expression of *SLC2A1* and the related transporter *SLC2A3* compared to M-CSF-derived MDMs (Fig. 6.20C). Therefore, as demonstrated by the schematic depicted in Figure 6.20D, GM-CSF induces the expression of several glycolysis-associated enzymes and transporters, and supports the notion that enhanced inflammatory responses by these cells may be driven by an enhanced metabolic profile and switch to glycolysis. This is of direct relevance to IL-1β production, as O'Neill and colleagues demonstrated that a glycolytic switch in macrophages drives HIF-1α-dependent IL-1β production [343].

A recent publication by Medzhitov and colleagues demonstrated that IL-10 stimulation of macrophages has a profound effect in suppressing immune responses by inhibiting glycolysis, GLUT-1 expression, and mitochondrial mass. Indeed, they hypothesised that altered mitochondrial dynamics might be a principle mediator of these effects given the observation that IL-10 induced changes in the side scatter of these cells by flow cytometry, which correlated with their intracellular complexity. Given the observed reduction in side-scatter of intestinal macrophages in the absence of ILCs, we hypothesised that GM-CSF may support an enhanced inflammatory profile and glycolysis in these cells by altering their mitochondrial fitness, either by inducing changes in mitochondrial fission/fusion or in mitochondrial maintenance within cells. To address this, GM-CSF primed macrophages were stained for mitochondria using MG, a cell membrane-permeable dye that can be used on live cells. Encouragingly, we found that GM-CSF treatment enhanced total mitochondrial mass in BMDMs (Fig. 6.21), suggesting this may be a facet of intestinal macrophage biology regulated directly by ILCs.

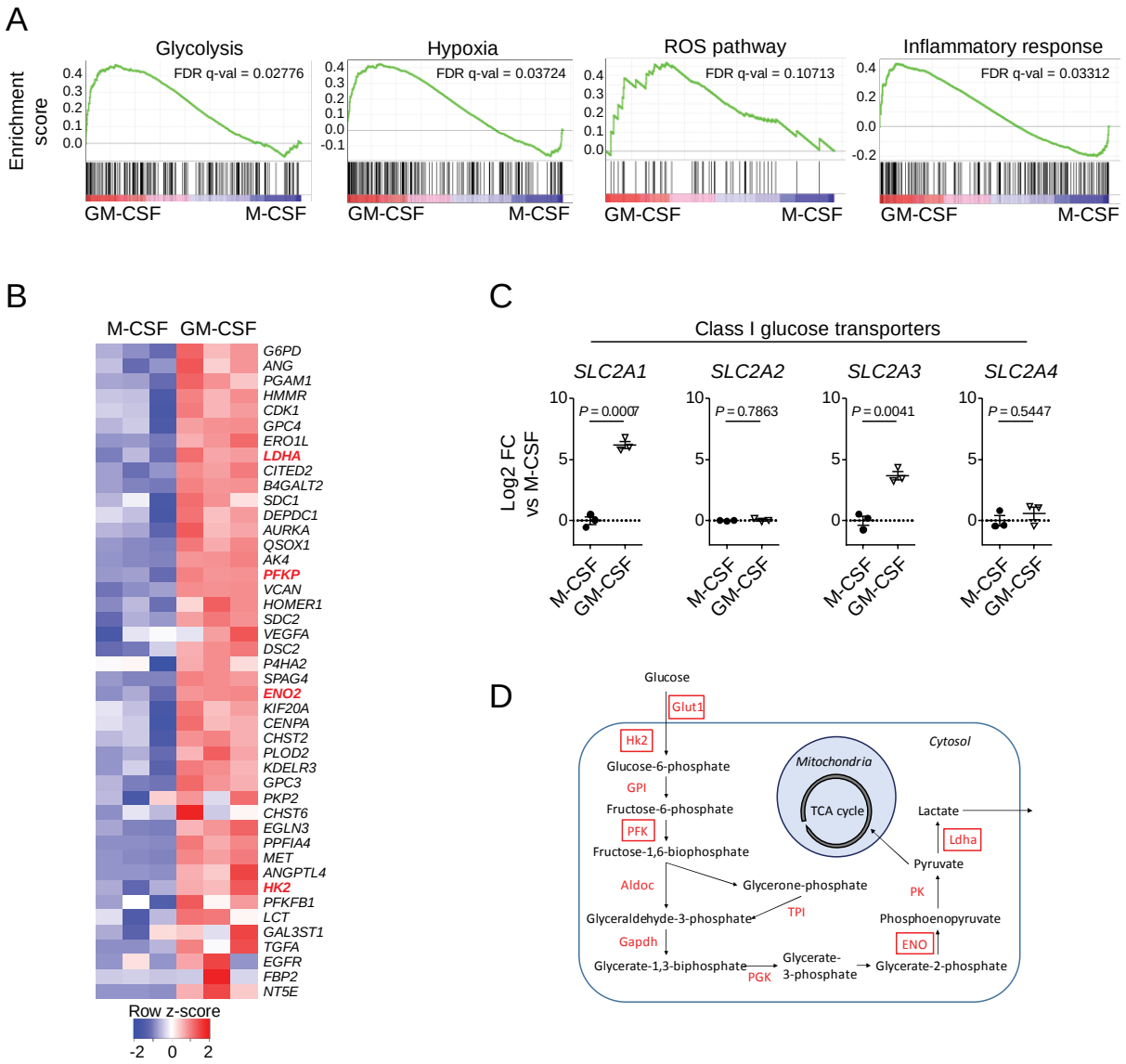

Figure 6.20. GM-CSF induces glycolysis in macrophages. (A) GSEA of inflammatory pathways in M-CSF and GM-CSF-derived human MDMs (GSE71253). (B) Heatmap showing core enrichment glycolysis genes in GM-CSF-derived MDMs. (C) Expression analysis of glucose transporters in M-CSF versus GM-CSF-derived MDMs. (D) Schematic showing the glycolysis pathway, with enzymes in red, and boxes surrounding GM-CSF-induced genes. *P* values were calculated using limma with multiple correction using BH.

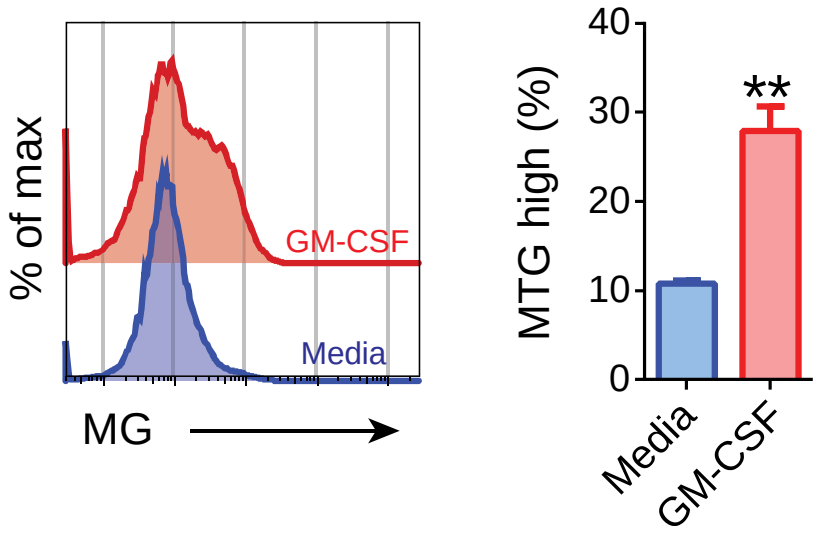

Figure 6.21. GM-CSF priming increases macrophage mitochondrial mass. Flow cytometric analysis of mitochondrial mass after 48 h stimulation of BMDMs with 20 ng/ml GM-CSF using MG. Data represent a single experiment. $n = 3$ per group. P values were calculated using the parametric Student's t test. ** $P < 0.01$.

6.10. IL-1β-mediated cross-talk between macrophages and ILCs

ILC3s are known to be regulated by macrophage-derived cytokines, most notably IL-23 and IL-1β. Given the role of GM-CSF in directly promoting production of both cytokines by macrophages, we hypothesised that this cross-talk is essential *in vivo* for potentiating macrophage-mediated immunity.

While analysis of GM-CSF expression by ILC subsets demonstrated high expression by both ILC3s and ILC2s within the SI (Fig. 6.22A), all of which are depleted with anti-CD90.2 IgG antibodies, IL-1R1 and IL-23R expression was predominantly restricted to ILC3 subsets (Fig. 6.22B). Therefore, IL-1β and IL-23-mediated cross-talk would only affect cytokine production by ILC3s. Incubation of flow-sorted RORγt$^+$ ILC3s with various ILC-activating cytokines demonstrated that only IL-1β stimulation, of those tested, induced GM-CSF expression by ILC3s (Fig. 6.22C), with negligible effect of IL-23A or IL-7. In contrast, IL-22 production was induced by both IL-23 and IL-1β, although the effect was greatest for IL-1β. Given the role of GM-CSF in promoting LPS-induced IL-1β and IL-23 production, this suggests that during a local inflammatory response, macrophage cytokine production in response to TLR stimulation would induce GM-CSF expression by ILCs, resulting in a positive feedback loop of enhanced macrophage activation, increased cytokine production and glycolysis, and greater ILC3-derived GM-CSF. Furthermore, this would support secondary induction of ILC3-derived IL-22 production, with implications for GM-CSF indirectly in the regulation of the epithelium.

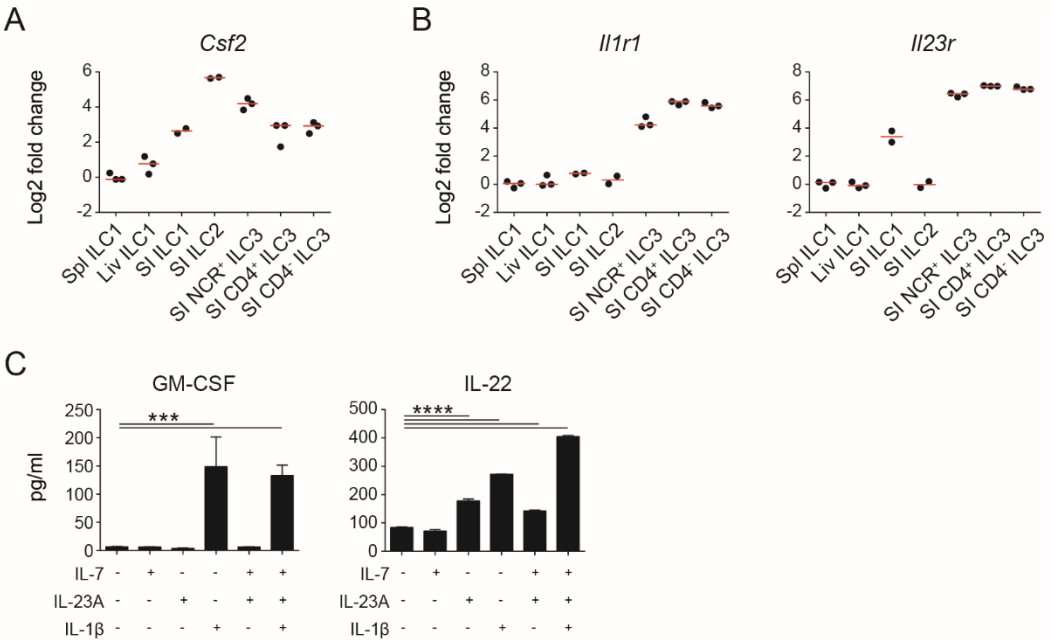

Figure 6.22. IL-1β induces GM-CSF production by ILC3s. (A) Analysis of *Csf2* expression by ILC subsets. (B) Analysis of inflammatory cytokine receptors by ILC subsets. (C) ELISA showing GM-CSF and IL-22 production by flow-sorted small intestinal ILC3s in response to cytokine stimulation. Data are representative of two independent experiments. *n* = 3 per group. *P* values were calculated using a one-way ANOVA. *** $P < 0.001$; **** $P < 0.0001$.

6.11. Discussion

6.11.1. ILCs are essential for defence against *Citrobacter rodentium* infection and promote DSS-induced colitis

ILC3s are enriched within the GI tract, where they have a major role in the regulation of function and tone of the epithelium. This is primarily achieved through the production of IL-22, which has been shown to be protective in models of colitis and epithelial barrier disruption, including Graft-versus-host disease [361], [399]–[402], [544], [577], [594]. This is achieved via the induction of anti-microbial peptides, regulating epithelial cell glycosylation, and stem cell turnover, amongst others [397], [575], [576]. ILC3s are also critically implicated in the pathogenesis of colitis and colorectal cancer, however, both in mice and humans [292], [398]. While IL-22 can promote tumorigenesis as well as induce pathogenic epithelial production of IL-18 [403], [404], [583], [625], [626], ILC3-derived IL-17A, and GM-CSF have been shown to support innate colitis and mucosal inflammation via the recruitment and activation of monocytes and granulocytes, respectively [297], [414], [415], [585]. In general, however, the mechanisms by which ILCs regulate proximal immune cells is poorly understood.

Here, we have investigated the role of ILCs in two models of intestinal pathology via a depletion strategy in which *Rag2*-deficient mice were treated with an anti-CD90.2 IgG depleting antibody. Specifically, we have demonstrated that in the absence of ILCs, *Rag2*-deficient mice exhibit enhanced caecal CFUs and systemic dissemination in a model of *C. rodentium* infection. Despite enhanced bacterial burden, intestinal MHC-II^{hi} macrophages exhibited an unresponsive phenotype, with reduced total colonic macrophage numbers, reduced pro-IL-1β expression, and defective MHC-II upregulation following the maturation of monocytes.

Strikingly, we observed a very similar macrophage phenotype in *Rag2*-deficient mice following an acute course of DSS-induced colitis. ILC depletion resulted in reduced macrophage numbers, inhibited pro-IL-1β expression, and resulted in significantly diminished global levels of IL-1β, TNF, IL-22 and GM-CSF. In this model, neutrophil recruitment to the inflamed colon was also significantly reduced, and may reflect the overwhelming inflammatory response induced by DSS: while trending towards a decreased in *C. rodentium* infection, DSS-induced inflammation may augment defects in the ability to recruit neutrophils. Curiously, the initial clinical phenotype of ILC-depleted mice was striking. While colon length was shorter and stool score increased in the absence of ILCs, consistent with worse inflammation, colon weight was significantly reduced in the absence of ILCs. This may reflect the differential effect of ILC-derived mediators on different aspects of intestinal physiology. For example, the enhanced rectal bleeding may result from impaired barrier repair in the absence of ILC-derived IL-22, amphiregulin, and IL-17A. However, the absence of GM-CSF may lead to defective leukocyte recruitment and activation to the inflamed tissue, resulting in reduced abscess formation, and

tissue thickening and destruction. Therefore, GM-CSF would be pathogenic in this instance, mirroring observations made by Powrie and colleagues in models of anti-CD40 and T cell transfer-driven colitis [297].

These results support a hypothesis that ILC-derived mediators are responsible for detrimental inflammation in DSS-induced acute inflammation but are required for the containment and possibly killing of *C. rodentium*, through the direct recruitment and regulation of tissue-resident macrophages. Macrophage phenotype here was determined by flow cytometric analysis, limiting our investigation to relatively few key macrophage genes. We would hypothesise that ILCs may regulate anti-microbial mechanisms in macrophages, such as ROS production that contribute to bacterial clearance. From these results, however, we cannot determine whether this is the case, or identify the molecules involved in regulating macrophage phenotype. Finally, it would be of interest to determine whether exogenous administration of depleted macrophage cytokines, such as IL-1β, is sufficient to restore the inflammatory response in anti-CD90.2 IgG-treated mice.

Rag2-deficient mice are an excellent tool to study the role of ILCs *in vivo*, given the absence of confounding adaptive T helper cell subsets and the greater contribution of ILC-derived cytokines to the local milieu. Furthermore, the use of *C. rodentium*, which is known to induce type 17 responses, also contributes to the specific investigation of ILC3-mediated immunity. However, these mice are a tool, and whether or not these results translate to immunocompetent mice with adaptive immune cells is not clear. However, mechanisms to specifically deplete ILC3s in WT mice are not readily available. It should be noted that GM-CSF is known to be expressed by Th17 cells and, under certain conditions, B cells [367], [406]. Therefore, it is likely that GM-CSF secretion would be compensated to a certain extent, although the contribution of different cell types in WT mice to colonic GM-CSF secretion has not been investigated here. In this study, we demonstrated a reduction in MHC-II expression by macrophages, which is not possible to study in *Rag2*-deficient mice given the absence of T cells. However, these mice would allow for elegant experiments involving the transfer of commensal-specific T cells without the confounding effects of local T cell responses.

6.11.2. GM-CSF depletion improves DSS-induced colitis severity and dampens macrophage cytokine production

Macrophages fail to develop appropriately within the colon in the absence of ILCs, suggesting that ILC-derived factors are required for adequate macrophage recruitment, maintenance and activation locally. Given the pan-ILC depletion strategy, several candidate cytokines exist with known roles in the regulation of macrophage responses, including IL-17A, GM-CSF, and IFNγ. Furthermore, we demonstrated significant expression of monocyte-recruiting chemokines by ILC3s, including CX3CL1, CCL3 and CXCL10. Whether ILCs are a major source of

chemokines within the intestine has not been investigated, with studies largely focusing on cytokine production. Therefore, it is intriguing whether this facet of ILC biology contributes to the coordination of immune cells during inflammation.

Of those cytokines investigated, GM-CSF was most notably diminished following anti-CD90.2 IgG administration in both models of colitis. Intestinal macrophages express high levels of GM-CSF receptor subunits, with lower expression of IL-17R and IL-22R, while i*n vitro* GM-CSF priming of BMDMs greatly augmented IL-1β secretion in response to LPS. *C. rodentium* is a Gram-negative bacterium, so LPS was used as a reasonable model of the TLR engagement elicited by this bacterium. However, it would be of interest to use heat-killed *C. rodentium* in future to see if these effects are synonymous. Therefore, the macrophage phenotype observed here suggested a central role for this cytokine. Of interest would be the role of GM-CSF on the generation of ROS. ROS are required for the anti-microbial activity of macrophages, and may support clearance appropriate *C. rodentium* clearance in the presence of ILCs. Furthermore, ROS is involved in the activation of the inflammasome, raising the possibility that GM-CSF-primed macrophages secrete mature IL-1β through a ROS-dependent mechanism [342].

GM-CSF depletion in DSS-induced colitis phenocopied certain features of pan-ILC depletion, including improved disease progression, reduced neutrophil recruitment, and impaired macrophage cytokine production. However, these effects were not as striking as pan-ILC depletion. This may be expected given the pleiotropic roles played by several ILC subsets in the GI tract. GM-CSF may cooperate with ILC3-derived IL-17A or ILC1-derived IFNγ, both of which are known to promote macrophage anti-microbial activity and cytokine production *in vivo* [585], [597]. Alternatively, the GM-CSF dosing strategy used here may not be optimal. Two doses of 0.125 mg anti-GM-CSF IgG as used here was employed by Powrie and colleagues in murine models of anti-CD40 colitis [297]. However, studies investigating the role of GM-CSF in DSS-induced colitis and *C. rodentium* infection used a double concentration [416]. The higher dosing strategy remains to be repeated, and should help resolve whether GM-CSF-independent mechanisms contribute to ILC-mediated intestinal inflammation and macrophage activation.

It is noteworthy that monocyte recruitment was completely unaffected by GM-CSF depletion, and supports an GM-CSF-independent role for ILCs in the recruitment of these cells to inflamed tissues. As well as chemokine production, ILCs could function indirectly via other local haematopoietic or non-haematopoietic cells. For example, IL-22 signalling in epithelial cells can drive the production on inflammatory mediators, such as IL-18 [583]. It is possible that epithelial activation may also drive the production of chemokines, such as CCL2, that contribute to immune cell recruitment.

6.11.3. GM-CSF regulates macrophages metabolism and inflammatory cytokine production

Several studies have highlighted a role for GM-CSF in the priming of monocyte inflammatory responses *in vivo*, and the activation of BMDMs *in vitro*. GM-CSF drives a switch to glycolysis and the expression of GTPases that support inflammatory cytokine production [410], [411]. The latter mechanism is not sufficient to explain differences in pro-IL-1β expression *in vivo*, however. It is possible that GM-CSF drives transcriptional expression of IL-1β, as well as promoting its cleavage. In support of this, we also observed a striking induction of LPS-induced IL-23 expression by BMDMs *in vitro* following GM-CSF priming. As its production is fundamentally different to IL-1β, this cannot be explained by differences in assembly of the inflammasome. This is in agreement with a study by O'Neill and colleagues demonstrating that macrophage IL-1β production upon LPS challenge was dependent on a glycolytic switch and the succinate-mediated stabilisation of the TF HIF-1α [343]. Indeed, GSEA of human MDMs stimulated with GM-CSF demonstrated enrichment of the glycolysis pathway, with increased expression of several key glycolysis genes, including LDHA, HK2, and the glucose transporter GLUT1 (SLC2A1). Analysis of these genes in macrophages from ILC-depleted mice or anti-GM-CSF IgG-treated mice would help to decipher the contribution of this pathway to macrophage fitness *in vivo*. However, previous studies have demonstrated that *in vitro* GM-CSF-driven IL-1β production is HIF-1α-independent [411]. Furthermore, why the induction of chemokine expression was less pronounced is unclear, but this may reflect a diminished requirement for metabolic reprogramming for these cytokines, similar to TNF. Therefore, the complete mechanistic underpinning of GM-CSF-driven inflammatory responses are not clear.

Medzhitov and colleagues recently demonstrated that regulation of macrophage fitness, metabolism and cytokine production is mediated by intrinsic IL-10 signalling [425]. In the absence of this signalling, macrophages accumulate damaged mitochondrial through the suppression of mitophagy, resulting in elevated inflammatory responses driven by IL-1β. The similarities with the observed phenotype presented here have led us to speculate that GM-CSF may play a role in regulating mitochondrial fitness. One might postulate that GM-CSF promotes a switch to glycolysis and the accumulation of mitochondria, resulting in an elevated capacity for cytokine production. In line with this, we demonstrated increased mitochondrial mass in GM-CSF-primed macrophages.

The mechanism for this accumulation, however, is not clear. It may be that GM-CSF regulates the clearance of mitochondria through a similar suppression of mitophagy. The use of a membrane potential-sensitive mitochondrial dye would help to determine whether this is the case. Alternatively, GM-CSF may regulate the fusion and fission of mitochondria. Furthermore, we hypothesised that GM-CSF may regulate the production of ROS that contribute to bacterial killing in models of *C. rodentium* infection. ROS is also implicated in the

regulation of the inflammasome, promoting mature IL-1β secretion, as observed by Medzhitov and colleagues [342], [425]. Therefore, GM-CSF-induced ROS production could have a central dual role in microbial killing and inflammatory cytokine production.

6.11.4. IL-1β-mediated cross-talk between macrophages and ILCs

Whether or not the above *in vitro* observations can be recapitulated by co-culture of ILC3s and macrophages remains an ongoing area of investigation. Notably, GM-CSF is expressed by several ILC subsets, including ILC2s and ILC3s. Therefore, pan-ILC depletion using anti-CD90.2 IgG antibodies is likely to prevent designation of phenotype to particular cell types. However, we demonstrated that IL-1R1 and IL-23R expression is largely confined to ILC3s. Furthermore, culture of ILC3s with IL-1β significantly induced GM-CSF production, demonstrating that not only can GM-CSF priming promote IL-1β production, but that IL-1β is essential for ILC3-driven GM-CSF production. Intriguingly, IL-22 production could be induced by both IL-1β and IL-23, both of which are regulated by GM-CSF. This raises the possibility that GM-CSF activation of macrophages has secondary effects of the epithelial barrier via the induction of both IL-23 and IL-1β by macrophages following antigen encounter. To this end, the investigation of IL-22 mediated mechanisms, such as anti-microbial peptide production, epithelial cell turnover and STAT3 signalling in epithelial cells, in the absence of ILC3-derived GM-CSF would be of interest. Therefore, ILC3-macrophage cross-talk is essential for the functionality of both cell types, and drives a positive feedback loop with potential secondary effects on surrounding cells.

In vivo studies investigating the IL-1β dependence on GM-CSF are lacking, but these results suggest that IL-1β blockade should suppress its own production in these circumstances via a block on GM-CSF secretion. This could be investigated *in vitro* through ILC3-macrophage co-cultures in the presence of blocking anti-IL-1R1 IgG and anti-GM-CSF IgG antibodies.

Finally, a consistent feature of DSS-induced colitis and *C. rodentium* infection in the absence of ILCs is the reduction in local macrophage numbers. We have already speculated that this may be due to an impairment in monocyte maturation and survival of cells within the LP. However, it is also possible that ILC-derived chemokines are required for the maintenance of these cells within tissues. Strikingly, we observed significant expression of *Cx3cl1*, the chemokine that binds to CX3CR1. As such, ILCs-derived CX3CL1 may contribute to the maintenance of $CX3CR1^{+}$ MNPs within the colon. How chemokine production by ILCs is regulated is largely unknown, but this may also participate in the ILC-macrophage cross-talk: ILCs promote the maintenance and activation of macrophages within the LP, which in turn reinforce GM-CSF and chemokine production by ILCs via IL-1β for their own survival.

6.11.5. Summary

In conclusion, we have demonstrated that ILC depletion significantly alters the activation state of intestinal macrophages in separate models of colitis (Fig. 6.23). This results in detrimental bacterial outgrowth in a model of *C. rodentium* infection but protection from overwhelming inflammation in DSS-induced colitis. These effects are in part mediated by the absence of GM-CSF, which primes macrophages for glycolysis and inflammatory cytokine production in response to microbial stimulation. ILC-macrophage cross-talk underlies these observations, as GM-CSF promotes macrophage IL-1β and IL-23 production, while these cytokines are essential for ILC3-derived GM-CSF and IL-22 secretion *in vitro*, respectively. Furthermore, ILC3s may potentially promote macrophage maintenance within the LP via secretion of chemokines, including CCL3, CXCL10, and CX3CL1. A significant number of additional experiments will be required to clarify the cellular mechanisms at play here, as will be discussed in the final chapter.

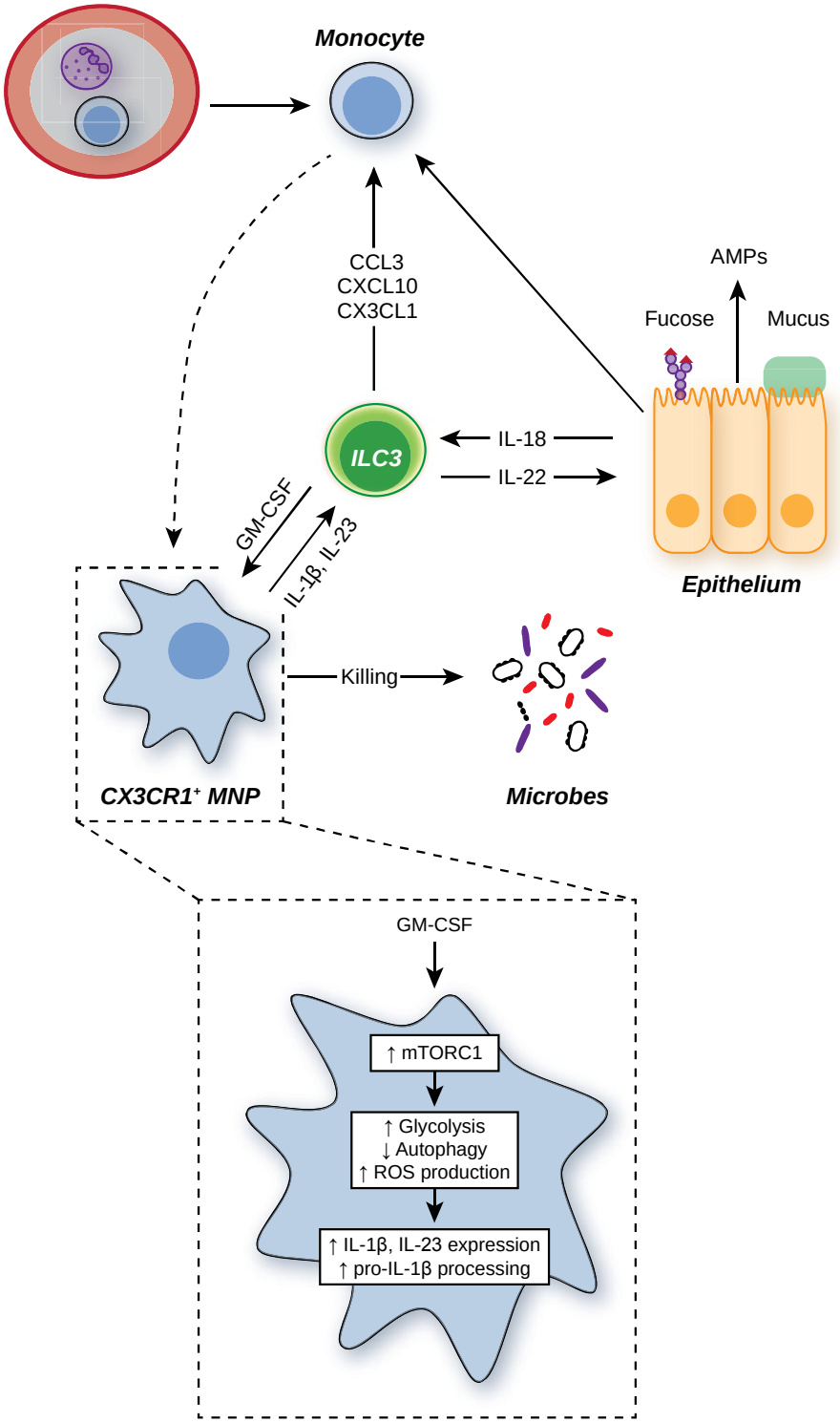

Figure 6.23. Chapter 6 graphical summary. ILCs regulate intestinal macrophage activation, mediated through production of GM-CSF. GM-CSF induces macrophage mTORC1 activation and glycolysis, resulting in enhanced cytokine production. GM-CSF may potentially contribute to cytokine production through the suppression of autophagy and the subsequent accumulation of pro-IL-1β and mitochondria. GM-CSF-induced cytokines support ILC3 activation via ILC-macrophage cross-talk, with subsequent implications for the activation of the intestinal epithelium via production of IL-22. Whether ILC3s contribute directly to the recruitment of monocytes to the inflamed mucosa is not clear, but is supported by observations that ILC3s express high levels of the chemokines CCL3, CXCL10, and CX3CL1, all associated with monocyte and macrophage recruitment.

7. Conclusion and future directions

7.1. IgG and FcγR signalling in intestinal inflammation

Despite the observation that mucosal anti-commensal and auto-antibodies are frequently identified in patients with IBD [241], [429], [430], there has been little widespread acceptance that IgG ICs might play a pathogenic role in UC. The dogma that mucosal humoral immunity is purely dominated by IgA, however, is changing, with the observation that homeostatic anti-commensal IgG generation has important implications for intestinal immune development, vertically-transmissible immunity to neonates, and defence against infection [284], [477], [479]. The association of the FcγRIIA-R/H131 variant (rs1801274) as the most significant non-HLA genetic variant with UC in a Japanese GWA study [156] supports a pathogenic role for IgG in this disease, driven by engagement of FcγRs. FcγRIIA-R131 profoundly reduces affinity for IgG and confers protection against UC, a finding confirmed in a subsequent meta-analysis of IBD GWA studies [154].

Attempts to mechanistically link intestinal IgG and FcγR function have focused on *in vitro* stimulation assays that interrogate the generic production of pro-inflammatory cytokines [430], with limited effort to ensure relevance to *in vivo* findings in UC. Our results highlighted a strong association between FcγRIIA and IL-1β in UC colonic biopsies, which was subsequently borne out by *in vitro* stimulations with IgG IC. Furthermore, global and macrophage FcγR A/I ratios were significantly skewed towards activation during inflammation, lowering the cellular activation threshold and allowing macrophages to be more readily activated by IgG IC. Most importantly, *in vivo* manipulation of macrophage A/I ratios was sufficient to dictate the severity of IL-1β-driven intestinal inflammation via the activation of mucosal Th17 immune responses. Blocking this pathway proved beneficial in *Fcgr2b*-deficient mice, and adds to the growing literature into the role of IL-1β in mediating detrimental intestinal inflammation [279], [296]. Therefore, our data implicate anti-commensal IgG and FcγR signalling as a previously unappreciated driver of this pathway *in vivo*.

Our data have therapeutic implications, identifying antibody and activating FcγR receptor signalling as potential therapeutic targets in UC. Previously, we discussed the limited number of studies targeting IgG and B cell responses in IBD, with reports of clinical improvement following IVIG [437]–[439] and acute responses to rituximab administration [440]. Furthermore, small molecule SYK inhibitors used to block FcγR signalling have demonstrated significant benefit in the treatment of autoimmune diseases, such as RA [226]. However, such broad approaches have significant off-target effects, and more targeted therapeutics are required. Here, we have demonstrated that manipulation of FcγRIIB expression on macrophages is sufficient to modulate disease severity in colitis. Therapeutic strategies targeting FcγRIIB function have been suggested, and is indeed one of the major modes of

action of IVIG [227]. As well as its role in the suppression of activating FcγR signalling, an FcγRIIB agonist could influence B cell activation and drives plasma cell apoptosis, regulating both the generation and effector functions of anti-commensal IgG [106], [107]. It is possible that these therapies could supplement novel drugs targeting Th17 immunity, including IL-23 and IL-1β blockade, to improve clinical remission rates. Recently, modification of the IgG glycome has been attempted to drive pro-inflammatory IgG to an anti-inflammatory state. IgG sialylation is essential for the function of IVIG [137], and enforced sialylation converts arthritogenic IgG into inhibitors of CIA [223]. Whether this is possible *in vivo* for the manipulation of human disease is not known, but opens the way for a personalised approach to redirect host detrimental mechanisms to a beneficial endpoint.

We observed significant variability in the IgG anti-commensal response in UC patients, which correlated with disease activity. As we enter the era of personalised medicine, it may be that UC patients could be stratified by FcγRIIA genotype and anti-commensal IgG response to tailor FcγR-centric therapeutic strategies in a targeted manner. Due to CNV, *FCGR* genes are often excluded from GWA studies, and little literature exists as to how other FcγR SNPs may contribute to IBD susceptibility. For example, the high-affinity FcγRIIIA-V158 variant is associated with susceptibility to RA and ITP [165]–[167] and may contribute to IBD susceptibility in a manner analogous to FcγRIIA-H131. Alternatively, FcγRIIB dysfunction arising from the T232 polymorphism could similarly promote chronic IgG-driven intestinal inflammation [145], [161], as we demonstrated in *Fcgr2b*-deficient mice. Indeed, it is possible that IBD susceptibility in FcγRIIB-T232 homozygous individuals would be driven by exacerbated myeloid responses to IgG, as well as elevated anti-commensal IgG responses as a result of B cell dysregulation and plasma cell survival. Other than IBD susceptibility, IgG responses may contribute significantly to prognosis: exacerbated IgG responses may well arise secondarily to the onset of inflammation in IBD, but subsequently drive a therapeutically refractive state through the perpetuation of inflammation. In this case, how FcγR variants contribute to IBD prognosis would be of great interest. Our results here suggest that following the onset of inflammation, perhaps through barrier defects in UC, IL-1β-driven inflammation could be enhanced by local IgG. Subsequently, inflammatory alterations in the IgG glycome, such as desialylation, could be driven by IL-1β-mediated Th17 responses [178], resulting in the positive reinforcement of inflammatory networks.

While also associated with anti-commensal IgG, the association of FcγR variants with CD susceptibility is much less robust that that observed in UC [154], [263]. CD susceptibility is centred on defects in pathogen recognition and clearance, with notable genetic associations with *NOD2* and *ATG16L1* [234], [247]–[250]. Reduced NOD2 function, an intracellular receptor for peptidoglycan, results in bacterial outgrowth, dysbiosis, and the loss of host-commensal mutualism [252], [253]. Mutations in *ATG16L1*, an autophagy gene, promote its

degradation and reduce autophagy, resulting in ER stress, epithelial dysfunction, and aberrant cytokine production [249], [254]–[258]. It is notable, therefore, that FcγRs are required for mucosal bacterial clearance in mice and humans [78], [283], [523] and FcγRIIA-R/R131 homozygosity is associated with invasive pneumococcal disease [627]. Therefore, defective activating FcγR signalling may contribute to defective invasive microbial clearance in patients with CD.

Secondary considerations regarding this differential association arise from the inflammatory networks contributing to CD and UC pathogenesis [318]. IFNγ is associated with CD [319], [320], but FcγR-TLR co-stimulation of M1 macrophages does not have a significant effect on the augmentation of inflammatory responses [74], [75]. In contrast, IL-4-primed human macrophages respond potently to FcγR-TLR co-stimulation by the production of Th17-inducing cytokines, including IL-1β. IL-4 is associated with UC pathogenesis, and may contribute to the robust association with FcγRIIA.

In summary, our study reveals the mechanisms by which anti-commensal IgG, acting via activating FcγRs cross-linking on intestinal macrophages, augments inflammation by inducing Th17 immunity via IL-1β. This sheds light on the observed genetic association of FcγRIIA polymorphisms with UC and identifies novel therapeutic targets.

7.2. The function of FcγRs on ILC3s

ILCs are a newly identified group of lymphocytes that are specialised for the rapid production of T cell-associated cytokines prior to the induction of adaptive immunity [12]. These cells are enriched at mucosal sites, with a growing body of literature demonstrating that ILC3s have important roles in regulating epithelial cell function via the production of IL-22, and the coordination of local immune responses through GM-CSF and IL-17A secretion [285], [297], [396], [544], [575], [577], [583], [585]. Given the rapid kinetics of ILC activation, studies have largely focused on the role of PRR-induced cytokines derived from macrophages, DCs, and epithelial cells in the activation of these cells. However, cross-talk with adaptive immunity is important in certain circumstances, with ILC-mediated antigen presentation demonstrated to suppress T cell activation within GALT and induce T cell responses in the spleen and lung [543], [554], [587], [589]. Furthermore, ILC3s have also been implicated in the activation of B cell responses via production of BAFF and expression of CD40L within the spleen [51].

The results presented in chapter 5 demonstrate that ILC3s and ILC1s are also capable of responding to IgG via their expression of FcγRIII, the canonical low affinity activating FcγR in mice. This presents a novel paradigm for ILC function, demonstrating that ILCs can integrate signals from adaptive immunity to regulate their activation state. The investigation of FcγR function on the closely related NK cell has had widespread implications for our understanding of anti-tumour and anti-viral immunity. FcγRIII cross-linking induces ADCC and IFNγ secretion

that contribute to target cell death and a potent anti-viral response [102], [104], [105]. Whether the investigation of FcγR signalling in helper ILCs will result in similar advances remains to be seen. We have demonstrated here that FcγR signalling on ILC3s induces the expression of several transcriptional regulators and chemokine receptors that promote ILC3 phenotype and function, e.g. AHR. Furthermore, we observed an increase in MHC-II expression. However, whether FcγR signalling has similar roles on ILC1s remains to be deduced. Given their similarity to NK cells, FcγRIII cross-linking on ILC1s may well induce IFNγ secretion, with implications for disorders in which ILC1s accumulate, such as colitis [597]. In future, it would be of interest to flow-sort intestinal ILC1s, ILC3s and NK cells from uninflamed and DSS-inflamed mice and perform transcriptomics analysis. This would give us a widespread understanding of inter-ILC variation in cellular responses to FcγR cross-linking and also how inflammation alters this response. This may be of particular importance given the upregulation of FcγRIIB during colitis.

Whether human helper ILC subsets express FcγRs is not clear. Several studies have suggested that FcγRIII expression in humans is confined to NK cells, and is absent from ILC subsets [597], [628]. However, a widespread characterisation of FcγR genes across ILC subsets is lacking, while this repertoire may potentially change during inflammatory conditions. We are currently in the process of flow-sorting human ILC3s to profile FcγR expression ourselves by qPCR.

At present, the functional consequences of ILC3-intrinsic FcγR signalling *in vivo* are still to be characterised in great detail. A recent study demonstrated that maternal IgG was required for the appropriate development of ILC3s within the neonatal GI tract [479]. The reason for this requirement was not clear. We would hypothesise that ILC3-intrinsic FcγR signalling mediated by maternal IgG may induce a transcriptional programme in this cells that promotes their survival locally, mediated by induction of key ILC3-associated TFs and chemokine and cytokine receptors. As such, IgG is not only relevant during chronic inflammatory conditions, but also in early life prior to the development of a fully-fledged intestinal humoral response. In future, we hope to resolve whether FcγR signalling on ILC3s can indeed support ILC3 function *in vivo* in colitis models and during development. To this end, we are currently in the process of generating RORγt-Cre FcγRIIB-floxed mice. It may be hypothesised that FcγRIIB suppresses the pro-ILC3 phenotype induced by activating FcγR signalling, promoting the transition to the inflammatory ILC1 subset. If this were the case, these mice may display elevated numbers of ILC3s during colitis and improved clinical disease via IL-22-dependent barrier reinforcement and the attenuation of IFNγ-dominated responses.

Furthermore, we hope to determine how AHR-mediated mechanisms may promote IC-induced IL-22. For example, do FcγRIIB-deficient ILC3s express higher levels of AHR and AHR-target genes associated with their elevated production of IL-22? These studies are currently

underway, and we hope will help to discern the functional importance of ILC3 FcγR signalling *in vivo*.

7.3. ILC-macrophage crosstalk mediates intestinal immune defence

In the final chapter of this thesis, we have attempted to elucidate the mechanisms by which ILC3s may regulate other tissue-resident immune cells in two separate models of intestinal inflammation. We have demonstrated that in the absence of ILCs, via a CD90.2 depletion strategy, mice are susceptible to *C. rodentium* infection, with greater CFUs at day 7 post-infection, but significantly protected from detrimental inflammation in an acute model of DSS-induced inflammation.

Mechanistically, we have focused on the role of GM-CSF in the coordination of macrophages, as in the absence of ILCs, these cells exhibit an unresponsive phenotype, with reduced cytokine production and total numbers, correlating with the absence of this cytokine *in vivo*. GM-CSF has previously been shown to mediate accumulation of monocytes and granulocytes within the inflamed colon and regulate DC maintenance [297], [414]–[416], [506]. However, the mechanistic underpinning of these observations was not addressed. GM-CSF is known to promote an M1-like phenotype in macrophages *in vitro*, with enhanced glycolysis and boosted IL-1β production [72], [410], [411]. However, we suspect that these mechanistic insights do not entirely account for the mechanism of GM-CSF function. A recent study demonstrated that IL-10 suppresses macrophage function by the suppression of MTORC1, resulting in an increase in autophagy, mitochondrial clearance, and a reduction in IL-1β expression [425]. The GM-CSF-mediated glycolytic switch was also dependent on MTORC1. Therefore, we hypothesise that ILC3-derived GM-CSF induces the activation of MTORC1 and the subsequent suppression of autophagy. Not only would this support a higher mitochondrial capacity, but also inhibit the clearance of pro-IL-1β, which has been shown to be regulated by autophagy [629]. Therefore, the observed reduction in pro-IL-1β expression by ILC-depleted macrophages *in vivo* could be due to a reduction in *Il1b* expression and an increase in engulfment of pro-IL-1β. Furthermore, the observed increase in CFUs *in vivo* in the absence of ILCs could result from impaired anti-microbial capacity by macrophages, with defective ROS production. As ROS production can also mediate inflammasome activation, we hypothesise that GM-CSF priming may induce ROS to induce bacterial killing and pro-IL-1β cleavage.

In future, we hope to carry out mechanistic studies *in vitro* into the role of GM-CSF on macrophage function. Specifically, does GM-CSF induce MTORC1 activation, the suppression of autophagy, and the accumulation of mitochondria and pro-IL-1β transcripts? Furthermore, can GM-CSF promote ROS production that could both contribute to bacterial killing and induce activation of the inflammasome? These studies would help to further our understanding of the mechanistic roles played by GM-CSF in the immune response other than

in haematopoiesis. In addition, we hope to carry out co-cultures between ILC3s and macrophages to determine whether a GM-CSF-IL-1β cross-talk circuit can promote similar observations in the absence of exogenous cytokines. This would be the first demonstration, as far as we are aware, that ILC3s can directly regulate the metabolic state and inflammasome activation of macrophages. Finally, we would like to translate these observations to our models of intestinal inflammation. We have already demonstrated that GM-CSF depletion suppresses IL-1β expression by macrophages *in vivo*, but is this related to a wider phenotype of macrophage dysfunction and does this have implication for defence against *C. rodentium* infection? Furthermore, we previously hypothesised that IL-22 may be diminished by GM-CSF blockade as a result of decreased IL-1β and IL-23 responses by macrophages *in vivo*. Could IL-22-mediated mechanisms, particularly on the epithelial barrier, be similarly defective in the absence of GM-CSF that contribute to defective bacterial clearance? These outstanding experiments are currently in progress, and will hopefully shed some light on the complex ILC-macrophage dynamic occurring within the GI tract.

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9. Appendix

Table S1. Gene lists for GSEA and hierarchical clustering

Dataset	Genes
FcγR phagocytosis	*AKT1, AKT2, AKT3, AMPH, ARF6, ARPC1A, ARPC1B, ARPC2, ARPC3, ARPC4, ARPC5, ARPC5L, ASAP1, ASAP2, ASAP3, CDC42, CFL1, CFL2, CRK, CRKL, DNM1, DNM1L, DNM2, DNM3, DOCK2, FCGR1A, FCGR2A, FCGR2B, FCGR2C, GAB2, GSN, HCK, INPP5D, LIMK1, LIMK2, LYN, MAP2K1, MAPK1, MAPK3, MARCKS, MARCKSL1, MYO10, PAK1, PIK3CA, PIK3CB, PIK3CD, PIK3CG, PIK3R1, PIK3R2, PIK3R3, PIK3R5, PIKFYVE, PIP4K2B, PIP5K1A, PIP5K1B, PIP5K1C, PLA2G4A, PLA2G4D, PLA2G4F, PLA2G6, PLCG1, PLCG2, PLD1, PLD2, PPAP2A, PPAP2B, PPAP2C, PRKCA, PRKCB, PRKCD, PRKCE, PRKCG, PTPRC, RAC1, RAC2, RAF1, RPS6KB1, RPS6KB2, SCIN, SPHK1, SPHK2, SYK, VASP, VAV1, VAV2, VAV3, WAS, WASF1, WASF2, WASF3, WASL*
FcγRIIB cross-linking	*ABL2, ABTB2, ACSL1, ADAR, ADM, ADPRH, AKR1C4, AMD1, ANKS1A, APOBEC3G, ARAP2, ARF6, ARHGAP25, ARID5A, ASCL2, ATF3, ATF6, ATG12, AXL, B4GALT5, BAK1, BARD1, BAZ1A, BIRC2, BMPR2, BRCA2, BST2, C1orf38, C22orf28, C2orf27A, CACNA1A, CASP10, CCL13, CCL2, CCL7, CCL8, CCR1, CD2AP, CD86, CDK17, CEACAM1, CGGBP1, CHSY1, CKAP4, CLDND1, CNTN1, CSTF3, CTNS, CXCL11, CXCL9, DACH1, DHX15, DHX34, DIO3, DNAJA1, DRAP1, DUSP6, DYNLT1, EED, EIF4G3, ELF4, ENPP2, ETV6, EVPL, FAM190B, FAS, FBXO7, FPR2, FTSJD2, FUT4, FZD2, GCA, GRIK2, GTF2B, H3F3B, HCK, HEG1, HIRA, HIVEP2, IFI35, IFI44, IFI44L, IFIT1, IFIT2, IFIT3, IFITM1, IFITM3, IGF2BP3, IL6, IL7, IRF7, IRF9, ISG15, ISG20, KBTBD2, KIAA0226, KIAA0754, KIAA1109, KRT76, LAG3, LAMP3, LCP2, LGALS9, LIG4, LILRB1, LMNB1, LMO2, LOC100131510, LRRC42, LYN, MACF1, MALT1, MEIS3P1, MFN1, MTMR6, MX1, MX2, MYL12A, MYO1B, N4BP1, NAMPT, NAPA, NASP, NDRG2, NRIP1, NUP62, OAS1, OAS2, OASL, P2RY6, PAPD7, PLN, PLSCR1, PPFIBP1, PPP2R2A, PPP3CC, PPP4R1, PSMA4, PTPN2, PVRL2, RAB14, RABGAP1L, RALB, RASGRF1, RBBP6, RBCK1, RBM34, RBMS1, RBPJ, RERE, RGL1, RIF1, RIN2, RIPK1, RNF144A, RNF19B, RSAD2, S100G, SASH1, SBNO2, SERPINB1, SETD1B, SFT2D2, SLC10A1, SLC31A2, SMAD3, SOCS1, SP100, SP110, SRGAP2, SRSF4, SSB, SSTR2, ST3GAL5, STAT2, STBD1, STK17B, STRN, TDRD7, TGM1, TLK2, TMEM110, TNFRSF1A, TOP1, TOP1P2, TOR1B, TRAFD1, TRIM14, TRIM21, TRIM25, TRIM38, TWF1, UBE2S, USPL1, XAF1, XIAP, ZC3HAV1, ZFP36, ZNF200, ZNF207*
Cytokines	*AIMP1, CCL1, CCL11, CCL12, CCL17, CCL19, CCL2, CCL20, CCL21A, CCL21B, CCL21C, CCL22, CCL24, CCL25, CCL27A, CCL27B, CCL28, CCL3, CCL4, CCL5, CCL6, CCL7, CCL8, CCL9, CD40LG, CD70, CKLF, CLCF1, CMTM2B, CMTM3, CMTM4, CMTM5, CMTM6, CMTM7, CMTM8, CNTF, CSF1, CSF2, CSF3, CTF1, CTF2, CX3CL1, CXCL1, CXCL10, CXCL11, CXCL12, CXCL13, CXCL14, CXCL15, CXCL16, CXCL2, CXCL3, CXCL5, CXCL9, CYTL1, D17WSU104E, DKK3, EBI3, EDA, EDN1, ERBB2IP, FAM3B, FAM3C, FASL, FBRS, FLT3L, GH, GM10591, GM12597, GM13271, GM13272, GM13275, GM13276, GM13277, GM13278, GM13279, GM13283, GM13285, GM13306, GM1987, GM2506, HC, IFNA12, IFNA13, IFNA14, IFNAB, IFNE, IFNG, IFNK, IFNZ, IK, IL10, IL11, IL12A, IL12B, IL13, IL15, IL16, IL17A, IL17B, IL17C, IL17F, IL18, IL19, IL1A, IL1B, IL1F6, IL1F8, IL1F9, IL1RN, IL2, IL21, IL22, IL23A, IL25, IL27, IL33, IL4, IL5, IL6, IL7, IL9, ILTIFB, LIF, LTA, LTB, MIF, NAMPT, NRADD, OIT1, OSM, PF4, PPBP, PRLH, SCG2, SCGB3A1, SECTM1A, SECTM1B, SOCS5, SPP1, SPRED2, SPRED3, THPO, TNF, TNFSF10, TNFSF11, TNFSF12, TNFSF13, TNFSF13B, TNFSF14, TNFSF15, TNFSF18, TNFSF4, TNFSF8, TNFSF9, TRIP6, TSLP, TXLNA, VAV3, WNT1, WNT2, WNT4, WNT5A, WNT7A, XCL1*

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